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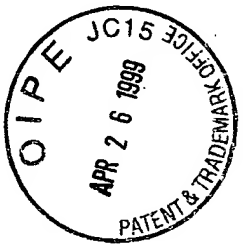

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I, DAVID DANIEL CLARKE , ASSISTANT DIRECTOR PATENT SERVICES, hereby certify that the annexed are true copies of the Provisional specification and drawing(s) as filed on 9 May 1996 in connection with Application No. PN 9765 for a patent by PHARMA PACIFIC MANAGEMENT PTY LTD filed on 9 May 1996.

I further certify that the annexed documents are not, as yet, open to public inspection.

WITNESS my hand this Sixteenth
day of April 1997

DAVID DANIEL CLARKE
ASSISTANT DIRECTOR PATENT SERVICES

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant(s): PHARMA PACIFIC MANAGEMENT PTY LTD
A.C.N. 055 778 348

Invention Title: STIMULATION OF HOST DEFENCE MECHANISMS

The invention is described in the following statement:

STIMULATION OF HOST DEFENCE MECHANISMS

This invention relates to methods of stimulation of defence mechanisms against pathological conditions in a host mammal by administration of interferon via the oropharyngeal cavity. In particular, the invention is applicable to methods of treatment of neoplastic disease and parasitic disease, and neurological disorders such as multiple sclerosis.

BACKGROUND OF THE INVENTION

Alpha interferons are used widely for the treatment of a variety of haematologic malignancies including hairy cell leukaemia, chronic myelogenous leukaemia, low grade lymphomas, cutaneous T-cell lymphomas, and solid tumours such as renal cell carcinoma, melanoma, carcinoid tumours and AIDS-related Kaposi's sarcoma (Gutterman, J.U., Proc. Natl. Acad. Sci. USA, 1994 91 1198-1205). Antitumour effects are usually seen at high dosage levels, often of the order of tens of millions of units of interferon-α (IFN-α), administered by parenteral injection. Interferon-β (IFN-β) is licensed for clinical use in treatment of relapsing-remitting multiple sclerosis and of chronic infection with Hepatitis B virus. Interferon-α and Interferon-β are both type I interferons. Although a number of routes of administration, including intravenous, subcutaneous, intramuscular, topical, and intralesional injection, are commonly employed for the administration of type I interferons, the oral route has not been generally used, because interferons are proteins which are considered to be inactivated by proteolytic enzymes and which are not absorbed appreciably in their native form in the gastrointestinal tract. Indeed a number of studies have failed to detect interferons in the blood following oral administration (Cantell and Pyhäla, J. Gen. Virol., 1973 20 97-104; Wills *et al*, J. IFN Res., 1984 4 399-409; Gilson *et al*, J. IFN Res., 1985 5 403-408).

It has been shown that inducers of interferon are able to protect mice against experimental infection with *Plasmodium berghei* malaria, and that this protection is much more effective against sporozoite-induced infection than against infection induced by blood forms of the parasite (Jahiel *et al*, Science, 1986 16 1802; Nature, 1968 220 710; Amer. J. Trop. Med. Hyg., 1969 18 823). Mice injected intraperitoneally or intravenously with interferon in pooled serum of Newcastle disease virus-infected mice were protected against sporozoite-induced *Plasmodium berghei* malaria. However, interferon from rabbit serum was ineffective. Protection was obtained when the interferon was injected during the pre-erythrocytic phase of parasite development (*ie*. three hours before or up to about 40 hours after sporozoite inoculation [Jahiel *et al*, Nature 1970 227 1350-1351]).

There have been a number of anecdotal reports of efficacy of low doses of interferon administered as a nasal spray or as an oral liquid formulation in the treatment of a variety of viral conditions, particularly influenza. However, in most of these reports the interferon preparations used were relatively crude. A placebo-controlled trial of high dose intranasal interferon for treatment of rhinovirus infection showed that the treatment was effective, but that there was a significant incidence of side-effects (Hayden *et al*, J. Infect. Dis., 1983 148 914-921). Similarly, although a number of studies including two randomized double-blind clinical trials (Douglas *et al*, New Engl. J. Med., 1986 314 65-80; Hayden *et al*, New Engl. J. Med., 1986 314 71-75) have demonstrated the efficacy of nasally administered high dose recombinant interferon-α2 in protecting exposed subjects against rhinovirus infections, these studies provided no evidence for a systemic effect.

More recently a series of patent specifications has described the use of low doses of orally-administered

interferon of heterologous species origin for the treatment of infectious rhinotracheitis ("shipping fever") in cattle, and of feline leukaemia, and also treatment of other conditions, for enhancement of efficiency of vaccines; for improving the efficiency of food utilisation; and for prevention of bovine theileriosis. See U.S. Patent No. 4,462,985, Australian Patent No. 608519, Australian Patent No. 583332 and U.S. Patent No. 5,215,741 respectively. In addition U.S. Patent Application No. 141621 discloses the use of interferon in this way for treatment of side-effects of cancer chemotherapy or radiotherapy. In these specifications, the interferon used was human interferon-α prepared by the method of Cantell, administered in phosphate buffered saline, at a dose of 0.01 to 5 IU per pound body weight. While these specifications suggest that such low doses of interferon administered to the oropharyngeal mucosa, preferably in a form adapted for prolonged contact with the oral mucosa, may be efficaceous for treatment of a wide variety of conditions including cancer, the experimental evidence for conditions other than shipping fever, feline leukaemia, canine parvovirus and theileriosis is largely anecdotal. In particular, no properly controlled trials of this treatment in any animal model for human cancers are presented.

More recent studies on the effects of very low doses of interferon administered by the oral or oropharyngeal mucosa have been reviewed (Bocci, Clin. Pharmacokinet., 1991 21 411-417; Critic. Rev. Therap. Drug Carrier Systems, 1992 9 91-133; Cummins and Georgiades, Archivum Immun. Therap. Exp., 1993 41 169-172). It has been proposed that this type of treatment is particularly useful for treatment of HIV infection, and can at least improve quality of life in AIDS patients (Kaiser *et al*, AIDS, 1992 6 563-569; Koech *et al*, Mol. Biol. Ther., 1990 2 91-95). However, other reports indicate that such

treatments provide no clinical benefit. A Phase I study of use of oral lozenges containing low doses of interferon for treatment of hepatitis B has also been reported (Zielinska *et al*, Archiv. Immunol. Therap. Exp., 1993 41 241-252).

In contrast, International Patent Application No. WO 95/27499 by Brigham and Women's Hospital showed that Interferon-β administered by gastric intubation could at least partly suppress development of autoimmune diseases such as Type I diabetes and autoimmune arthritis in well-recognized animal models, if given before the inducing antigen. Interferon-β given by this route or intraperitoneally in conjunction with intragastric "bystander" antigen was even more effective in inducing tolerance.

This suggests that interferon-β is effective in enhancing the induction of oral tolerance, rather than in inducing either humoral or cellular responses to exogenous antigen.

We have now surprisingly found that Type I interferon administered to the oropharyngeal cavity by the intranasal/oral route was effective in protecting mice against challenge with highly metastatic tumour cells. The quite exceptional nature of these results, together with the fact that very few substances exhibit activity against these very aggressive tumours, indicates that administration of interferon to this site is useful in the treatment of cancer.

SUMMARY OF THE INVENTION

In its general aspect the invention provides a method of stimulating host defence mechanism(s) against a pathological condition in a mammal, comprising the step of administering an effective dose of a type I interferon to the oropharyngeal cavity of a mammal in need of such treatment. Interferons so administered are able to contact the lymphoid tissue present in the oropharyngeal cavity to

induce host resistance to systemic disease via a mechanism(s) which does not involve either the direct action of the exogenously administered interferon or the induction of endogenous interferons as evidenced by the absence of increased levels of interferon induced-proteins in the peripheral blood mononuclear cells of the interferon treated animals.

The treatment may be prophylactic or therapeutic, or may be directed at ameliorating the symptoms of disease. For prophylaxis against neoplastic disease, it is contemplated that the method of the invention will be applicable where the subject to be treated is at high risk of cancer because of genetic predisposition, because of occupational or other exposure to ionizing radiation or to carcinogenic or cancer-potentiating chemicals, such as asbestos, nitrosamines, or tobacco smoke, or because the individual is known to have a risk factor such as possessing an inactive or mutated tumour suppressor gene such as p53 or Rb.

While it is to be clearly understood that the invention is applicable to any pathological condition in which stimulation of host defence mechanisms is beneficial, preferably the pathological condition is a neoplastic disease, neurological disorder such as multiple sclerosis or a parasitic infection. It will also be understood that for the purposes of the invention a Type I interferon may be used either alone or in conjunction with another agent or treatment.

The invention generally provides a method of prevention, amelioration or treatment of disease in a mammal in need of such treatment, comprising the step of administering a Type I interferon of a sub-type selected from the group consisting of IFN-α, IFN-β, and IFN-ω to the oropharyngeal cavity of said mammal, wherein the dose of interferon is 200 to 20 x 10^6 IU day for a 70 kg adult. Preferably the dose is 10^4 to 10^6 IU/day. Also preferably

the interferon is administered twice a day in two equal doses.

In one embodiment, the invention provides a method of treatment of a neoplastic condition in a mammal, comprising the step of administering interferon as described above. The neoplastic condition may be metastatic cancer.

While the method of the invention may be used without concurrent treatment with other agnets, it is contemplated that this embodiment of the invention will be particularly useful in the following settings:

a) as adjuvant therapy, subsequent to surgery, reductive chemotherapy, or radiotherapy given by standard protocols.

b) for treatment of interferon-sensitive neoplasias, the method of the invention is utilised in conjunction with conventional chemotherapy or radiotherapy.

c) for treatment of interferon-resistant neoplasias, the method of the invention is utilised in conjunction with conventional chemotherapy or radiotherapy.

In setting a) the method of the invention is directed to prophylaxis against recurrence of neoplastic disease once remission has been induced by initial conventional treatment.

Both methods b) and c) above are directed at inducing and/or maintaining remission of disease. By "in conjunction with other treatment" is meant that the interferon is administered before, during and/or after the radiotherapy or other chemotherapy. The most suitable protocol will depend on a variety of factors, as discussed below.

In particular, it is contemplated that the method of the invention will preferably be used in conjunction with at least one other treatment selected from the group consisting of chemotherapy using cytostatic drugs, one or more other cytokines which have anti-cancer activity but

which have a different mechanism of action from that of interferon, anti-angiogenic agents, and agents which potentiate the activity of interferon. Preferably the second cytokine is interleukin-1 (IL-1), interleukin-2 (IL-2) or interleukin-12 (IL-12); preferably the angiogenesis inhibitor is AGM-1470; preferably the interferon-potentiating treatment is hyperthermia or arginine butyrate. The second cytokine may also be administered to the oropharyngeal cavity.

Preferred cytostatic drugs to be administered in conjunction with interferon include but are not limited to cyclophosphamide, cisplatin, carboplatin, carmustine (BCNU; N,N-Bis(2-chloroethyl)-N-nitrosourea), methotrexate, adriamycin, α-difluoromethylornithine, and 5-fluorouracil.

The neoplastic conditions susceptible to this method include but are not limited to cancers which respond to parenteral administration of high doses of IFN-α, such as haematological malignancies, *eg.* multiple myeloma, hairy cell leukaemia, or chronic myelogenous leukaemia, low grade lymphomas, cutaneous T cell lymphoma, solid tumours such as renal cell carcinoma and melanoma, carcinoid tumours, or AIDS-associated Kaposi's sarcoma.

In a second embodiment, the disease to be treated is malaria, and again a Type I interferon is administered as described above. The causative organism of the malaria may be *Plasmodium malariae, Plasmodium vivax, Plasmodium falciparum* or *Plasmodium ovale*. It is particularly contemplated that the method of the invention will protect against progression of malaria to the cerebral form.

It is contemplated that this embodiment of the invention will also be applicable to prophylaxis against malaria for travellers to high-risk areas.

In a third embodiment, the invention provides a method of treatment of neurological disorders such as multiple sclerosis, whether of the relapsing-remitting or the chronic progressive type, comprising the step of

administering a Type I interferon as described above.

The interferon to be used may be natural Type I interferon extracted from cell cultures, or may be of recombinant origin. Interferons from both of these sources are commercially available from a variety of sources. Either homologous or heterologous interferon may be used. Type I interferon from any of these sources is generically referred to herein as "interferon" (IFN).

For the purposes of the invention, the term "Type I IFN" also includes polypeptides or their fragments which have Type I IFN activity, or mutant forms of IFN in which sequence modifications have been introduced, for example to enhance stability, without affecting the nature of their biological activity.

Conventional vehicles and excipients for IFN may be used. The IFN formulation may comprise stability enhancers, such as glycine or alanine, as described in U.S. Patent No. 4,496,537, and/or one or more carriers, such as a carrier protein. For example, for treatment of humans pharmaceutical grade human serum albumin, optionally together with phosphate buffered saline as diluent, is commonly used. Where the excipient for IFN is human serum albumin, the human serum albumin may be derived from human serum, or may be of recombinant origin. Normally when serum albumin is used it will be of homologous origin.

The IFN may be administered by any means which provides contact of the IFN with the oropharyngeal cavity of the recipient. Thus it will be clearly understood that the invention is not limited to any particular type of formulation. The present specification describes administration of IFN deep into the nasal cavity; this may be achieved with nasal drops or sprays. Oral formulations which provide contact with the oropharyngeal cavity may also be used. It is not necessary that the IFN be swallowed, although it may be convenient for the recipient to do so. Thus the invention includes, but is not limited

to, liquid, spray, syrup, buccal, and nebuliser formulations. A person skilled in the art will recognise that for aerosol or nebuliser formulations the particle size of the preparation may be important, and will be aware of suitable methods by which particle size may be modified.

For the purposes of the animal experiments described in this specification, it will be clearly understood that the expression "intranasal/oral" or "intranasal plus oral" with reference to the route of administration of IFN is to be taken to mean administration of the IFN preparation deep into the nasal cavity so that it is rapidly distributed into the oropharyngeal cavity, *ie.* the mouth and throat of the recipient mammal, so as to make contact with the mucosa lining this cavity.

Within the specific dose ranges referred to above, the optimal treatment in any individual case will depend on the nature of the condition concerned, the stage of disease, previous therapy, other continuing therapy, the general state of health of the mammal *etc.*, and therefore will be at the physician's or veterinarian's discretion, bearing in mind all these circumstances.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows the effect of low dose intranasal/oral mouse IFN-α on survival of mice injected with highly metastatic Friend erythroleukaemia cells, using different doses and excipients;

Figure 1B shows the results of a similar experiment using recombinant human IFN-α 1-8;

Figure 2 compares the effect on survival of mice injected with Friend erythroleukaemia cells of murine IFN-α given by different routes;

Figure 3 shows the effect of frequency of intranasal/oral IFN treatment on the survival of mice injected with Friend erythroleukaemia cells;

Figure 4a compares the effect of different doses of IFN-α on expression of the MHC Class I antigen H-2K^d on the surface of monocytes following administration of IFN-α;

Figure 4b shows the effect of expression of this antigen on the surface of granulocytes;

Figure 5 shows the percentage of spleen cells expressing H2K^d antigen at different times following intranasal/oral administration of IFN-α;

Figure 6 shows expression of Ly 6A/E antigen on lymphoid cells at different times after intranasal/oral administration of IFN-α;

Figure 7 compares the effect of intranasal/oral and intraperitoneal IFN on the expression of 2'-5'-oligoadenylate synthetase in spleen cell; and

Figure 8 shows the activity of this enzyme in spleen cells at different times following intranasal/oral IFN;

Figure 9 shows IFN titres in serum of mice treated with ^{125}I-IFN-α 1-8 by different routes;

Figure 10 shows serum levels of ^{125}I-IFN-α 1-8 given by different routes up to 24 hr after administration; and

Figure 11 shows whole blood and serum levels of ^{125}I-IFN-α following intranasal/oral administration.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only to the following non-limiting examples and to the figures. The materials and general experimental methods used in these examples were as follows:

INTERFERONS AND INTERFERON FORMULATIONS

Mouse IFN-α/β

Mouse IFN-α/β (Mu IFN-α/β) was prepared from cultures of C243-3 cells induced with Newcastle disease

virus (NDV) and purified as described previously (Tovey *et al*, Proc. Soc. Exp. Biol. and Med., 1974 146 809-815). The preparation used in this study had a titre of 4×10^6 International Units (IU)/ml and a specific activity of 5×10^7 IU/mg protein as assayed on mouse 929 cells challenged with vesicular stomatitis virus (VSV) as described previously (Tovey *et al*, Proc. Soc. Exp. Biol. and Med., 1974 146 809-815). The preparation was standardised against the international reference preparation of murine IFN-α/β of the National Institutes of Health (NIH) (G-002-9004-5411).

Human IFN-α 1-8

Recombinant human IFN-α 1-8 (Hu IFNα 1-8; BDBB lot no. CGP 35269-1, Ciba-Geigy, Basel, Switzerland) was prepared and purified as described previously (Meister *et al*, J. Gen. Virol., 1986 67 1633-1643). The preparation used in this study had a titre of 70×10^6 IU/ml on homologous human WISH cells challenged with VSV as described previously (Tovey *et al*, Nature, 1977 267 455-457), and a titre on heterologous mouse L929 cells of 1×10^6 IU/ml. The preparation was standarized against both the NIH human IFN-α international reference preparation (G-023-901-527) and the NIH murine IFN-α/β standard (G-002-9004-5411). The specific activity of the IFN preparation was 2×10^8 IU/mg protein.

EXCIPIENT

Interferon preparations were diluted in phosphate buffered saline (PBS) containing bovine serum albumin (BSA). Bovine serum albumin fraction V (RIA grade; immunoglobulin free; Cat. no. A7888; Sigma; USA) was dissolved at a final concentration of 100 µg/ml in PBS (pH 7.4) and sterilized by filtration (0.2 µ, Millex-GV, Millipore, USA).

INTERFERON DELIVERY SYSTEM

Preliminary experiments showed that the application of 5 µl of crystal violet to each nostril of a normal adult mouse using a P20 Eppendorf micropipette resulted in an almost immediate distribution of the dye over the whole surface of the oropharyngeal cavity. Staining of the oropharyngeal cavity was still apparent some 30 minutes after application of the dye. Essentially similar results were obtained using ^{125}I-labelled recombinant human IFN-α 1-8 applied in the same manner. This method of administration was therefore used in all subsequent experiments.

FRIEND ERYTHROLEUKAEMIA CELLS

The IFN-α/β resistant clone, 3C18, of Friend erythroleukaemia cells (FLC) was obtained from Dr E. Affabris, Rome and is described in detail by Affabris *et al*, 1982 (Virology, 120 441-452). These cells were subsequently maintained by *in vivo* passage. Briefly, DBA/2 mice were inoculated by intraperitoneal (ip) injection (IP) with approximately 100 LD_{50} of 3C18 cells and one week later the tumour cells were harvested from the peritoneum of the mice, counted and other mice were again inoculated with 100 LD_{50} of 3C18 cells. This procedure was repeated for 60 to 100 passages. We have shown that the 3C18 cells used at the 60th to 100th *in vivo* passage are highly metastatic for the liver and spleen (Gresser *et al*, Int. J. Cancer, 1987 39 789-792). The phenotype of IFN resistance was confirmed routinely by cultivating the *in vivo* passaged cells *in vitro* in the presence of IFN-α/β (Belardelli *et al*, Int. J. Cancer, 1982 30 813-820).

ANIMALS

The mice used in this study were obtained from a specific pathogen-free colony (IFFA CREDO, France). They were housed in a specific pathogen-free animal facility at

the Institut Federatif CNRS at Villejuif according to EEC standards.

INTERFERON BIOASSAY

Interferon was assayed according to a conventional method. Briefly, samples (20 µl) were diluted in 80 µl of Eagle's Minimal Essential Medium (MEM) (Gibco, France) containing 2% heat-inactivated Fetal Calf Serum (FCS) (Gibco, France) and added to each well of a microtitre plate (Falcon, cat. no. 3072) using a multichannel micro-pipette (Finnpipette, Labsystem, 50-300 µl). WISH or L929 cells (2 x 10^4 cells/well) were added in 100 µl of MEM containing 2% FCS and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air (Forma 3029 CO_2 incubator). The cells were then examined for any signs of toxicity using an Olympus IM GLDW inverted microscope equipped with a 10X objective. Samples which did not exhibit detectable toxicity were then subjected to serial two-fold dilutions starting from an initial 1:10 dilution in a total volume of 200 µl of Eagle's MEM containing 2% FCS, by carrying forward 100 µl of diluted material with a multichannel micropipette, in a microplate containing 100 µl per well of fresh Eagle's MEM containing 2% FCS, Appropriate serial two-fold dilutions of the NIH human IFN-α reference standard (G-023-901-527) or the NIH Mu IFN-α/β reference standard (G-002-9004-5411) were also prepared. WISH or L929 cells (2 x 10^4 cells/well) in 100 µl of Eagle's MEM containing 2% FCS were then added to each plate where appropriate and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. The cell monolayers were then checked for any signs of toxicity and in the absence of any apparent toxicity, the culture was aspirated and replaced with 200 µl of Eagle's MEM containing 2% FCS containing 100 $TCID_{50}$ of VSV (2 x 10^{-4} VSV_{23} for WISH cells, or 10^{-5} VSV_{23} for L929 cells). The plates were then incubated overnight at

37°C in an atmosphere of 5% CO_2 in air. The cell monolayers were then examined for specific viral cytopathic effect using an Olympus IM ULWD inverted microscope. Interferon titres were determined from the reciprocal of the dilution which gave 50% protection against specific viral cytopathic effect, and are expressed in international reference units/ml (IU/ml).

Example 1 — Anti-Tumour Activity of Intranasal/Orally Administered Interferon-α in Mice Challenged with Friend Erythroleukaemia Cells

In order to establish whether IFN-α administered by the intranasal/oral (in/or) route increases the survival of mice challenged with highly metastatic FLC cells, groups of six 7-8 week-old male DBA/2 mice were challenged intravenously (iv) with 1 x 10^5 FLC on day 0.

Each group of mice was treated twice a day for 10 consecutive days by the in/or route with either 10^4 IU of a natural mixture of multiple murine IFN-α subtypes (Mu IFN-α) in 10 µl BSA-PBS, or with an equal volume of a mock IFN preparation, which was produced and purified in the same manner as the IFN preparation with the exception of the omission of the virus inducer. The mock IFN preparation did not exhibit detectable IFN activity when assayed in parallel with the purified Mu IFN-α preparation. The results are shown in Table 1.

Table 1

Group	Treatment	Day of Death	Mean day of death ± SE
1	Mock Mu IFN-α	9,9,10,10,10,10	9.6 ± 0.2
2	Mu IFN-α 10^4 IU	28,31,36>100>100	31.7 ± 2.3**

** Calculated for dead animals only

Intranasal/oral administration of 10^4 IU of Mu IFN-α twice a day dramatically increased the survival time of mice injected with FLC. In fact 2 of 5 mice were effectively cured as they were alive and well 100 days after challenge with 2 x 10^4 LD_{50} of FLC, despite cessation of IFN-α treatment after only 20 days.

Example 2 Larger Trial of Anti-Tumour Activity of Low Dose Intranasal/Oral Interferon-α in Mice Injected with Friend Erythroleukaemia Cells

In order to confirm the results of Example 1, a larger trial was performed. A total of 150 8 week-old female DBA/2 mice was challenged iv with 1 x 10^5 FLC (2 x 10^4 FLC LD_{50}) on day 0. Mice were treated with the type and dose of IFN indicated, administered by the in/or route in a 10 µl volume twice a day for 10 consecutive days. These were 10 mice in each treatment group. The results are summarised in Table 2, and illustrated in Figures 1A and 1B.

Table 2

Group	Treatment	Dose	Excipient	Mean day of death ± SE
1	None	-	None	9.5 ± 0.2
2	Mock Mu IFN-α	-	BSA/PBS	9.6 ± 0.2
3	Mu IFN-α	10^4 IU	BSA/PBS	*
4	Mu IFN-α	10^3 IU	BSA/PBS	11.6 ± 0.2
5	Mu IFN-α	10^2 IU	BSA/PBS	10.3 ± 0.2
6	Hu IFN-α 1-8	10^4 IU	BSA/PBS	18.2 ± 0.8
7	Hu IFN-α 1-8	10^3 IU	BSA/PBS	13.1 ± 0.8
8	HU IFN-α 1-8	10^2 IU	BSA/PBS	11.4 ± 0.5

IU : International reference units
BSA/PBS : 100 µg/ml of BSA in PBS pH 7.4
Mu IFN-α : natural mixture of murine IFN-α subtypes
Hu IFN-α : single recombinant human IFN-α isotype
* : Some of the animals in this group were still alive at 100 days post treatment

IFN-α administered by the in/or route exhibits a marked anti-tumour activity in mice challenged iv with FLC. Indeed, some IFN-treated mice can be considered to be cured, as they were still alive more than 100 days after inoculation of 100,000 FLC, in a system in which 4 to 5 FLC cells are sufficient to kill an animal (Figures 1A and 1B). In these figures "CN" denotes controls in which a modified excipient was used.

Both a pure preparation of a single recombinant IFN-α subspecies, IFN-α 1-8, and a natural mixture of multiple IFN-α subtypes, Mu IFN-α, exhibit marked anti-tumour activity in this model when administered by the in/or route, strongly suggesting that the observed anti-tumour activity of orally administered IFN preparations is indeed due to the IFN component of these preparations.

The highest dose of IFN tested (10^4 IU) was found to be the most effective, for both types of IFN used in the study.

The Mu IFN-α appeared to be more effective in this system than the Hu IFN-α 1-8.

Example 3 Effect of Route of Administration of Interferon on Anti-Tumour Activity

The effect of in/or administered IFN was compared with that of IFN given by conventional routes. Eight week-old female DBA/2 mice were challenged iv with 1 x 10^5 FLC (2 x 10^4 FLC LD_{50}) on day 0. Mice were treated twice a day for 10 consecutive days with 10^4 IU of Mu IFN-α (the optimal dose as determined in Example 2) administered by the route indicated. There were 6 mice in each treatment group, and in each case the excipient for the Mu IFN-α was BSA in PBS. The results are summarised in Table 3, and illustrated in Figure 2.

Table 3

Group	Treatment	Route	Mean day of death ± SE
1	None		9.6 ± 0.2
2	Mu IFN-α	Intranasal/oral	*
3	Mu IFN-α	Oral	18.5 ± 2.0
4	Mu IFN-α	Gastric	13.5 ± 1.3
5	Mu IFN-α	Subcutaneous	23.5 ± 1.6
6	Mu IFN-α	Intramuscular	23.7 ± 1.8
7	Mu IFN-α	Intravenous	25.0 ± 1.2
8	Mu IFN-α	Intraperitoneal	26.7 ± 4.1

IU : international reference units
BSA/PBS : 100 μg/ml of BSA in PBS pH 7.4
* : Some of the animals in this group were still alive. 100 days after inoculation of FLC cells.

It can be seen that the in/or route of administration is at least as effective as the parenteral routes commonly used in medical practice, such as iv, intramuscular (im), and subcutaneous (sc) injection, if not more effective. The in/or route was in fact as effective as ip injection, which is considered to be the most effective route in mice challenged iv with FLC. In contrast, administration of the same dose of IFN directly into the mouth appeared to be less effective than combined intranasal and oral administration, while introduction of IFN directly into the stomach of animals via a tube was considerably less effective.

Example 4 Effect of Frequency of Administration of Interferon

Increasing the frequency of administration of either preparation of IFN from once a day to twice a day

clearly increased the effectiveness of the treatment. This is illustrated in Figure 3. The dose of IFN was 10^4 IU.

This raises the possibility that even greater beneficial effects would be observed if the frequency of administration of IFN is increased further.

Example 5 Effect of Interferon Against Viral Infection

Intraperitoneal infection of mice with Encephalomyocarditis virus (EMCV) gives rise to a rapidly progressing fatal disease characterized by CNS involvement and encephalitis (Rueckart, R.R., in Virology, Fields ed., 705-738, 1985, Raven Press, New York). IFN-α has been shown to be effective in protecting mice against lethal EMCV infection when administered either prophylactically or even when administered after virus replication has already occurred in target organs (Finter, N.B.; Front. of Biol., 1973 2 135-147). Thus, in these studies we determined the effect of orally administered low doses of either natural or recombinant IFNs on the survival of mice injected with a lethal dose of EMCV, *ie.* in a very severe test of antiviral activity.

Encephalomycarditis virus strain (JH) was propagated on mouse L929 cells as described previously (Gresser *et al*; Proc. Soc. Exp. Biol and Med., 1968 127 491-496). The virus stock used in this study (EMC-I) had a titre of $5 \times 10^{8.62}$ $TCID_{50}$ on mouse L929 cells.

Treatment of mice with 200 or 2000 IU of Mu IFN-α/β or Hu IFN-α/β 1-8 via the in/or route increased the mean survival time of mice infected with a lethal dose of EMCV (300 LD_{50}). In all 6 experiments (Tables 5 and 6) Hu IFN-α/β 1-8 was as effective as Mu IFN-α/β.

The administration of the IFNs by the in/or route resulted in a statistically significant increase in the mean survival time in 6 of the 9 experiments carried out in this study. Furthermore, the overall trend was towards

efficacy in all the experiments undertaken.

Administration by the ip route (200 µl per injection) resulted in a significant increase in the mean survival time in 7 of the 8 experiments undertaken in this study using ip administration (Tables 4 and 5).

Table 4

Effect of Interferon on Survival of Mice Following Infection with ECMV

IFN	Dose IU/mouse	Excipient	Route	No. of mice	Day of Death	% survival	Mean day of death ± se
None		none		5	5,5,5,6,6	0	5.4 ± 0.2
Mu - α/β	10 000	BSA	ip	5	-----	100	-
Mu - α/β	2 000	BSA	ip	5	-----	100	-
Mu - α/β	200	BSA	ip	5	7,----	80	-
Mu - α/β	2 000	BSA	in/or	5	7,7,7,8,10	0	7.8 ± 0.6
Mu - α/β	200	BSA	in/or	5	7,10,---	60	-
Hu α 1-8	2 000	BSA	ip	5	-----	100	-
Hu α 1-8	200	BSA	ip	5	7,10,---	60	-
Hu α 1-8	2 000	BSA	in/or	5	5,7,11,--	40	-
Hu α 1-8	200	BSA	in/or	5	5,7,7,--	40	-

Interferon or excipient was administered once a day on days -2, -1, 0, +1, +2 and +3 in relation to EMCV challenge on day 0.

Table 5

The Effect of Combined Prophylactic and Treatment with IFN-α on the Survival of Swiss Mice Injected with 375 LD_{50} of ECMV

Treatment group	Dose	Route	Number of surviving animals / Per group (days post injection)										
			4	5	6	7	8	9	10	12	13	14	26
Untreated			5/5	5/5	1/5	0/5							
Mu IFN-α/β	200 IU	in/or	5/5	4/5	4/5	4/5	2/5	2/5	2/5	1/5	1/5	1/5	1/5
Mu IFN-α/β	2000 IU	in/or	5/5	5/5	5/5	4/5	3/5	2/5	2/5	2/5	1/5	1/5	1/5
Hu IFN-α 1-8	200 IU	in/or	4/4	4/4	3/4	3/4	1/4	1/4	1/4	0/4			
HU IFN-α 1-8	2000 IU	in/or	5/5	3/5	1/5	1/5	0/5						
Mu IFN-α/β	200 IU	ip	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Mu IFN-α/β	2000 IU	ip	5/5	5/5	5/5	5/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5
Mu IFN-α/β	10^4 IU	ip	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
Hu IFN-α 1-8	200 IU	ip	5/5	5/5	5/5	5/5	5/5	4/5	4/5	4/5	4/5	4/5	4/5
Hu IFN-α 1-8	2000 IU	ip	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5

Interferon was administerd twice a day on days -2, -1, 0, +1 and +2 in relation to EMCV challenge on day 0.

Table 6

Effect of Interferon Dose on Survival Following ECMV Infection

IFN	Dose IU/mouse	Route	No. of mice	Day of death	% survival	Mean day of death ± se
None		in/or	6	4,4,5,5,6,6	0	5.0 ± 0.4
Hu α 1-8	2 000	ip	5	15,17,---	60	-
Hu α 1-8	200	ip	5	9,10,16,17,17	0	13.8 ± 1.8
Hu α 1-8	2 000	in/or	5	5,6,7,7,21	0	9.2 ± 2.3
Hu α 1-8	200	in/or	5	5,6,6,7,9	0	6.6 ± 0.7

Interferon was administered twice a day on days 0, +1, +2, +3 and +4 in reaction to EMCV challenge on day 0

It is clear from the data in Tables 4 and 5 that whree ip and in/or administration can be directly compraed, the ip route to be marginally more effective (7/16 cases) or not significantly different (9/16 cases).

In animals treated with either Mu IFN-α/β or Hu IFN-α/β 1-8 administered by the in/or route, no significant difference was observed in the percentage of animals surviving infection for those animals treated with either 2000 IU or 200 IU of IFN, except in one case where greater efficacy was observed at the lower dose.

Our preliminary data suggest that the administration of IFN twice a day by either route does not appear to increase significantly the efficacy of treatment in protecting mice against infection (Table 6), relative to the administration of IFN once a day (Tables 4 and 5).

Example 6 — Relative Efficacy of Prophylactic and Therapeutic Treatment Against Oral Infection with EMCV

The mean survival time of mice treated in/or or ip with either 200 or 2000 IU of Hu IFN-α 1-8 was not significantly greater when IFN treatment was started on the day of infection with EMCV and continued for 4 days post-infection, than when IFN treatment was started 4 days prior to virus infection and stopped on the day of infection (Table 6).

The greatest increase in the percentage of mice surviving infection with a lethal dose of EMCV was observed in those experiments in which IFN therapy was started prior to virus infection and continued for 1 to 3 days post-infection (Table 4). Similar results were obtained for both Mu IFN-α/β and Hu IFN-α 1-8 administered either ip or by the in/or route (Tables 4 and 5).

Our results show that the administration of low doses of IFNs by the in/or route exerts a statistically significant increase in the survival of mice infected with

a lethal dose of EMC virus as determined by the log rank x^2 test (Mantel, 1966).

Example 7 Effect of Intranasal-Oral Interferon on Expression of Cellular Proteins

IFN-α is known to induce the expression of a number of cellular proteins following binding of the protein to its cell surface receptor. These proteins are thought to provide a useful marker of IFN action.

We evaluated the effect of IFN-α administered via the in/or route on the expression of three IFN-induced proteins, MHC class I antigens, Ly 6A/E antigen and 2'-5'-oligoadenylate synthetase.

Treatment of DBA-2 mice ($H\text{-}2K^d$) with up to 20,000 IU of Mu IFN-α by the in/or route did not significantly increase $H\text{-}2\text{-}K^d$ expression on peripheral blood lymphocytes, monocytes or granulocytes under conditions where as little as 20 IU of Mu IFN-α given ip markedly increased the expression of $H\text{-}2\text{-}K^d$ antigens on both peripheral blood monocytes (Figure 4a) and granulocytes (Figure 4b). Indeed, expression on monocytes was slightly suppressed. Furthermore, treatment of mice with 200 or 20,000 IU of either Mu IFN-α or Hu IFN-α 1-8 by the in/or route daily for 4 consecutive days did not significantly increase expression on splenocytes of MHC class I antigens determined at 1, 3, 6 and 10 days after the start of treatment. These results are shown in Figures 4 and 5 respectively.

Similarly, treatment of mice with up to 20,000 IU of IFN-α via the in/or route had no significant effect on the expression of Ly6 A/E antigens, the expression of which is markedly enhanced on the surface of a variety of lymphoid cells following parenteral treatment with type I IFN (Dumont *et al*; J. Immunol, 1986 137 201-210). Similar results were obtained with 200 or 20,000 IU of either Mu IFN-α or Hu IFN-α 1-8 via the in/or route. This is

illustrated in Figure 6.

Treatment of either Swiss or DBA/2 mice with as little as 20 IU of Mu IFN-α injected ip resulted in a marked increase in 2'-5'-oligoadenylate synthetase activity in both peripheral blood mononuclear cells and splenocytes. In contrast, in the same experiment treatment of mice with up to 20,000 IU of Mu IFN-α via the in/or route did not significantly increase the expression of 2'-5'-oligoadenylate synthetase activity. Furthermore, treatment with 200 or 20,000 IU of either Mu IFN-α or Hu IFN-α 1-8 by the in/or route had no significant effect on 2'-5'-oligoadenylate synthetase activity at any of the time points tested up to 10 days after the start of IFN treatment. These results are summarised in Figures 7 and 8 respectively.

Treatment of animals with up to 20,000 IU of Mu IFN-α either by ip injection or by in/or administration did not result in a consistent effect on any of the haematological parameters (erythrocyte count, haematocrit, haemoglobin content, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), reticulocyte count, total leukocyte count, number of lymphocytes, monocytes and polynuclear cells including neutrophils, eosinophils, and basophils) monitored on samples of peripheral blood in four independent experiments or on any of the blood chemistry parameters (blood urea nitrogen (BUN), creatinine, total lactate dehydrogenase (LDH), serum glutamate oxaloacetate transaminase (SGOT), serum phosphotransferase (SGPT), alkaline phosphatase, total protein and albumin content) monitored in three independent experiments.

Furthermore, treatment of mice with 200 or 20,000 IU of either Mu IFN-α or Hu IFN-α 1-8 by the in/or route daily for 4 consecutive days did not exert a consistent effect on any of the haematological or blood chemistry parameters determined at 1, 3, 6 and 10 days

after the start of treatment.

Considerable variation was observed, however, in the values obtained for a particular parameter, both between individual animals at a particular time point and between different time points within the same experiment. Variations were also observed in the values obtained from one experiment to another. Thus under these circumstances small differences in a given parameter between untreated and IFN treated animals would not have been detected. The values obtained for both the haematologic and blood chemistry determinations do, however, fall within the values previously published for normal mice.

These results suggest that of the three biomarkers tested, none adequately reflects the marked antiviral activity exerted by IFN-α administered by in/or route against a systemic virus infection, or the marked anti-tumour activity observed after intravenous challenge.

Example 8 Bioavailability of Interferon Following Intranasal/Oral Administration

In order to examine the bioavailability and pharmacokinetics of IFN, mice, which have the most favourable drug-blood volume ratio for such studies, were treated with a single high dose of recombinant IFN-α labelled to the highest specific radioactivity possible with ^{125}I.

A pure preparation of 70 x 10^6 IU of Hu IFN-α 1-8 was taken up in 1.4 mls of PBS, and iodinated as described by Mogensen *et al*, (Int. J. Cancer, 1981 28 575-582) using a modification of the chloramine-T method described by Hunter and Greenwood (Nature , 1962 194 495-496).

The ^{125}I-labelled Hu IFN-α 1-8 (lot no. CGP35269-1) exhibited a biological activity of 2 x 10^7 IU/ml when assayed on human WISH cells challenged with VSV and 1 x 10^6 IU/ml when assayed on mouse L929 cells

challenged with VSV.

Six to seven week-old female Swiss mice were injected iv, ip, or treated in/or with 2 x 10^7 IU equivalent to 1 x 10^6 murine IU of ^{125}I Hu IFN-α 1-8 (1.0369 x 10^7 cpm/mouse). At the time points indicated, three mice per group were sacrificed, blood was collected, and the volume determined. Kidney, liver, lung, spleen, and stomach/oesophagus were harvested, blotted, and weighed to a precision of ± 1.0 μg. The radioactivity of each sample was determined individually using a gamma counter. Whole blood was then separated by centrifugation (800 g x 10 min., 4°C), the serum was harvested, counted, and frozen at -80°C. The serum was then assayed for IFN content using a standard bioassay on both human WISH cells and on mouse L929 cells as described above. The radioactive material present in the samples of serum was then isolated by affinity immunoprecipitation and analyzed by SDS-PAGE.

Very high levels of radioactivity (> 2 x 10^6 cpm/ml) were detected in the peripheral blood of animals 5 min. after injection of 1.0369 x 10^7 cpm/mouse of ^{125}I-labelled Hu IFN-α 1-8 by iv bolus. The amount of radioactivity present in whole blood then declined progressively at 15 and 30 min. The levels of radioactivity detected in the peripheral blood of animals 5 min. after ip injection of 1.0369 x 10^7 cpm of ^{125}I Hu IFN-α-1-8 were approximately twenty fold lower than the levels detected following an iv bolus. The levels of radioactivity then increased progressively at 15 and 30 min. post-injection. The levels of radioactivity detected in the blood of animals at 5, 10 or 15 min. after the in/or administration of ^{125}I IFN-α 1-8 were significantly lower than those detected at a given time following ip injection of the same quantity of radiolabelled IFN. For all three routes of administration, higher levels of radioactivity were detected in serum than

in whole blood following in/or administration of ^{125}I-labelled IFN-α 1-8. The lower levels of radioactivity detected per ml of whole blood compared with the same volume of serum reflect the effectively larger volume of serum counted after removal of the cellular component of whole blood.

Samples of serum from all the mice in the study were assayed for the presence of biologically active IFN using a standard bioassay, as described above, and showed readily detectable levels of biologically active IFN in the serum of all the animals injected either iv or ip with ^{125}I Hu IFN-α 1-8 at all the time points tested. In contrast, no biologically active IFN was detected in the serum of any of the animals at any of the time points tested following the in/or administration of IFN, in spite of the presence of relatively high levels of radioactivity in the serum of these animals. This is shown in Figure 9.

In order to determine whether the radioactive material detected in the serum of animals treated with ^{125}I Hu IFN-α 1-8 does indeed represent native IFN, the samples were immunoprecipitated with protein A-G Agarose, in order to precipitate immunoglobulins present in the samples, treated with an affinity-purified polyclonal anti-IFN-α antibody, and further immunoprecipitated. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described above.

SDS-PAGE analysis of the radioactive material in serum following iv or ip injection of ^{125}I Hu IFN-α 1-8 revealed a single homogenous band migrating with an electrophoretic mobility identical to that of uninjected ^{125}I Hu IFN-α 1-8. The apparent molecular weight of the material was estimated to be approximately 20000 Daltons, which corresponds exactly to the molecular weight of native Hu IFN-α 1-8. In contrast, none of the samples of serum from mice treated in/or with ^{125}I IFN-α 1-8 contained any material with an apparent molecular weight similar to that

of native IFN, even though an identical quantity of radioactive material was loaded on to each gel.

The tissue distribution of radiolabelled material revealed very high levels of radioactivity in the kidneys, high levels in the liver, lung, and spleen of animals 5 min. after the iv injection of ^{125}I IFN-α 1-8. The level of radioactivity present in each of these four organs was then found to decrease progressively at 15 and 30 min. In contrast, the level of radioactivity in the stomach increased progressively at 15 and 30 min. to reach a level comparable to that present in the serum of animals 30 min. after an iv bolus.

Administration of ^{125}I IFN-α 1-8 by ip injection resulted in peak levels of radioactivity in all the tissues examined within 15 min., followed by a decline at 30 min. Similarly, in/or administration of ^{125}I Hu IFN-α 1-8 resulted in peak levels of radioactivity in all the tissues studied after 15 min. with some decline in the levels of radioactivity present at 30 min. The levels of radioactivity present in the stomach/oesophagus were an order of magnitude greater than those detected in any other organ following the in/or administration of ^{125}I-labelled IFN-α 1-8, and were markedly higher than the levels present in these tissues following parenteral administration of the same quantity of radiolabelled Hu IFN-α 1-8 by either the iv or ip routes.

Example 9 Pharmacokinetics of Interferon Following Intranasal/Oral Administration

For precise determination of the pharmacokinetics of Hu IFN-α 1-8, mice were treated iv, ip or in/or with 1.0369×10^7 cpm/mouse of ^{125}I-labelled Hu IFN-α 1-8, and the levels of radioactivity present in both whole blood and serum were determined at a series of time points over a 24 hour period.

The pharmacokinetic profile of ^{125}I-labelled Hu IFN-α 1-8 present in the blood of mice after an iv bolus closely followed a logarithmic clearance curve, as shown in Figure 10. This agreed with results of a previous study carried out in mice using a closely related molecule, recombinant human α A/D (Bgl) (Bohoslawed *et al*; J. IFN Res., 1986 6 207-213). The amount of bioavailable material, calculated from the area under the cure of concentration versus time, was also similar to that for human α A/D. A biphasic time-consuming clearance curve was observed following an iv bolus of ^{125}I IFN-α 1-8, which is characteristic of substances which are cleared through the kidneys, in agreement with the results of Example 9. The pharmacokinetics of ^{125}I-labelled IFN-α 1-8 following ip injection closely resembled those previously reported for IFNs administered im.

Readily detectable levels of biologically active IFN were present in the serum of all the animals following either an iv bolus (Figure 10) or ip injection (Figure 11) of ^{125}I-labelled IFN-α 1-8. Although antiviral activity could not be detected in the serum of animals following in/or administration of ^{125}I IFN-α 1-8, a statistically significant degree of protection against infection with a lethal dose of EMCV was nevertheless observed in these animals (Table 7).

Table 7

The Effect of a Single Treatment with ^{125}I-IFN-α on the Survival of Swiss Mice Injected with EMCV

Treatment group	Dose	Route	Number surviving animals / per group (Days post injection)									
			3	4	5	6	7	8	9	10	15	30
Untreated			6/6	4/6	2/6	0/6						
Hu IFN α 1-8	$1.4x10^5$ IU	in/or	6/6	5/6	4/6	3/6	1/6	1/6	1/6	1/6	1/6	1/6
Mu IFN-α/β	6.4×10^4 IU	in/or	6/6	5/6	5/6	4/6	3/6	2/6	2/6	2/6	1/6	1/6

Discussion

It is clear that for both types of IFN used in the tumour study the highest dose of IFN tested was the most effective. When considering these results it is important to remember that in the present study mice were injected iv with a massive dose of highly malignant and very aggressive tumour cells. It is probable that in mice injected with fewer tumour cells or in mice inoculated with a more slowly growing subcutaneous tumour, lower doses of IFN-α would have been more effective. Similarly, if higher doses of IFN-α had been used in the present experiments more of the animals would probably have been cured.

The Friend erythroleukaemia model constitutes a very severe preclinical test of anti-tumour activity, since FLC are highly malignant and metastasize to both the liver and spleen when injected iv. Indeed, results obtained using this model were the basis for the adoption of parenteral injection of IFN-α for treatment of human cancers. Thus, in all the experiments carried out in this study all the untreated animals and animals treated with control preparations died within 10 to 11 days. Injection of only 4 or 5 FLC cells will kill a mouse if no treatment is given. In contrast, some of the animals treated with murine IFN-α by the in/or route are still alive more than 100 days after inoculation of 10^5 FLC, and may be considered to be cured.

Indeed, judging from previous work, IFN-α administered by the in/or route appears to be more effective than cyclophosphamide, 5-fluorouracil, or methotrexate administered parenterally, which increase survival time by only a few days in animals injected with FLC (Gresser *et al*, J. Natl. Cancer Inst., 1988 80 126-131). Other drugs, such as cisplatin, vincristine, doxorubicin, bleomycin or etoposide are ineffective against this tumour (Gresser *et al*, J. Natl. Cancer Inst, 1988 80 126-131).

Similarly, IFN-α administered by the in/or route appears to be more effective against FLC than other cytokines such as IL-1β, IL-2 and TNF-α administered systemically, which exhibit very little activity in this model.

Previous work has shown that IFN administered parenterally is one of the most active anti-tumour drugs in this model, and that IFN therapy is effective even when initiated after tumour metastases are already present in the liver (Gresser *et al*, Intl. J. Cancer, 1987 39 789-792). The present results show that IFN administration by the in/or route is equally, or even more, effective.

Daily injections of IFN-α given together with a single dose of cyclophosphamide markedly increased the survival of lymphoma-bearing AKR mice compared to animals treated with either agent alone, when therapy was started after diagnosis of the lymphoma (Gresser *et al*, Eur. J. Cancer, 1978 14 97-99). Successful combination therapy using IFN-α/β and BCNU, *cis*-DDP (cisplatin), methotrexate, adriamycin, and α-difluoromethyl ornithine has also been reported in various pre-clinical animal tumour models. Combination therapy with 5-fluorouracil (5-FU) and IFN has also been reported to be of benefit in the treatment of metastatic colon cancer in man (Ernstoff *et al*, Journal of Clinical Oncology, 1989 7 1764-1765). There are, however, other studies which have reported a decreased anti-tumour activity when IFN therapy was combined with the use of cyclophosphamide (Marquet *et al*, Int. J. Cancer, 1983 31 223-226; Lee *et al*, Biochem. Pharmacol., 1984 33 4339-3443), adriamycin (Blackwill *et al*, Cancer Res., 1984 44 904-908), or 5-FU (Marquet *et al*, 1985 109 156-158), *ie.* precisely the same drugs which we and others have shown to exert a beneficial effect when used in combination with parenteral IFN therapy. Combinations between IFN and other chemotherapy agents can readily be tested using methods described herein.

Combined interleukin-1 (IL-1) and IFN-α/β therapy results in a synergistic anti-tumour effect in mice injected with FLC (Belardelli *et al*, Int. J. Cancer, 1991 49 274-278). The same treatment also exerts a marked anti-tumour effect against a metastatic variant (p11-R-Eb) of the Eb lymphoma, against which either agent alone is ineffective (Gabriele *et al*, Invasion Metastasis, 1993 13 147-162). Of all the cytokines tested, IL-1 was found to be the most effective when combined with parenteral type I IFN therapy.

Combination therapy with the angiogenesis inhibitor AGM-1470 given together with IFN-α/β resulted in a markedly increased anti-tumour effect compared to that observed with either agent alone (Brem *et al*, J. Pediatric Surgery, 1993 28 1253-1257).

It has been shown that hyperthermia enhances the anti-tumour action of IFN-α/β against the Lewis lung carcinoma (Yerushalmi *et al*, Proc. Soc. Exp. Biol. Med., 1982 169 413-415). Arginine butyrate has also been shown to potentiate the anti-tumour action of IFN-α (Chany and Cerutti, Int. J. Cancer, 1982 30 489-493).

Our results obtained in a well-defined preclinical model of an acute viral infection provided unequivocal evidence to support the "proof of principle" for the use of low dose in/or IFN for the therapy or prophylaxis of acute systemic viral infections in man, and show that both a natural mixture of multiple IFN-α subtypes and a single recombinant IFN-α isotype exert statistically significant antiviral activity in this model. Natural Mu IFN-α/β and Hu IFN-α 1-8 appeared to be equally effective when administered by the in/or route.

Comparison of the degree of protection obtaind when a given type and dose of IFN was administered by the in/or route compared to the results obtained following systemic administration (ip injection) showed that parenteral administration of IFN was in some cases

marginaly more effective, and in other cases no more effective, than in/or administration.

The results of the biomarker pilot study (Example 9) show quite clearly that none of the three biomarkers tested (MHC class I antigen, Ly6 A/E antigen, and 2'-5'-oligoadenylate synthetase activity) adequately reflects the very marked antiviral or antitumoural activity exhibited by IFN-α administered by the in/or route.

The contrast between the very marked increase in the expression of all three IFN-induced proteins observed in all the experiments undertaken following the ip injection of as little as 20 IU of IFN-α and the absence of any detectable effect following the administration of up to 20,000 IU of IFN-α via the in/or route is striking.

Although we cannot exclude the possibility that an effect on one or other of the biomarkers would have been observed at an earlier or intermediate time point, this seems to be unlikely, as IFN acts on the transcription of the genes coding for these proteins and thus one would not expect to see an effect on any of these biomarkers until a number of hours after IFN treatment.

Again, although we cannot exclude the possibility that a systemic effect on one of the other numerous IFN-induced proteins would have been observed following treatment with IFN-α by the in/or route, this seems unlikely, as this would imply a differential regulation of the expression of certain IFN-induced genes. It is entirely possible, however, that an effect on an IFN biomarker may be observed locally, for example, in nasal lymphocytes following administration of IFN-α via the in/or route.

In keeping with the absence of a detectable effect on the biomarkers studied, no consistent effect was observed on any of the haematological or blood chemistry parameters monitored during in/or IFN therapy, even in animals treated with up to 20,000 IU of IFN-α.

The results of the pharmacokinetics-bioactivity study show quite clearly that a statistically significant antiviral effect can be obtained following the in/or administration of a single dose of radiolabelled Hu IFN-α 1-8 under conditions where no circulating IFN can be detected in the peripheral blood, using methods of detection which are an order of magnitude more sensitive than those used previously. In keeping with these results the extent of the antiviral activity exerted by in/or administered IFN did not appear to follow a classical dose-response relationship. Thus, for both natural Mu IFN-α/β and Hu IFN-α 1-8 no statistically significant difference was observed in the extent of the antiviral effect observed when animals infected with a lethal dose of EMCV, were treated with either 200 or 2000 IU of IFN.

In previous bioavailability studies adequate measures were not taken to ensure retention of IFN in the oral cavity for a sufficient length of time to allow complete absorption. It is probable therefore, that the large majority of the IFN administered was degraded in the intestine prior to absorption. Thus, an optimal delivery system, specifically adapted to the mouse and allowing for sufficient retention of IFN in the oral cavity, was developed and validated prior to initiating the present bioavailability studies.

Readily detectable levels of radiolabelled material were found in both whole blood and serum of animals following in/or administration of ^{125}I-labelled IFN-α 1-8. These results contrast with the results of previous studies, which failed to detect IFN in the serum of animals even after the oral administration of large quantities of unlabelled IFN. However, the radioactive material detected in both whole blood and serum following in/or administration was biologically inactive. Furthermore, the results of SDS-PAGE analysis showed that this material was of low molecular weight, and most

probably reflected the absorption of degradation products following digestion of IFN in the stomach and small intestine. Analysis of the tissue distribution of radiolabelled material following in/or administration revealed markedly higher levels of radioactivity in the stomach than in any of the other organs tested. Our results show quite clearly that even though biologically active IFN was not absorbed following in/or administration, this treatment does nevertheless exert a statistically significant antiviral activity *in vivo*.

Without wishing to be bound by any proposed mechanism for the observed beneficial effect, our results suggest that in/or administered IFN exerts its effects against both tumour cells and viruses via a presently undefined novel mechanism, which does not involve a direct action of exogenously-administered IFN, or the induction of endogenous IFN. This is supported by the absence of detectable levels of circulatory IFN or of the three biomarkers tested. It appears that this mechanism may act at least partly by stimulation of the abundant lymphoid tissue surrounding the nasopharyngeal and oral cavities. Since we have shown that in/or IFN is at least comparable in efficacy to systemically-administered IFN, our results provide strong support for administration of IFN by the in/or route in the treatment or prophylaxis of neoplastic disease or acute viral infections. This could have important implications for the clinical use of IFN.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

PHARMA PACIFIC MANAGEMENT PTY LTD 9 May 1996

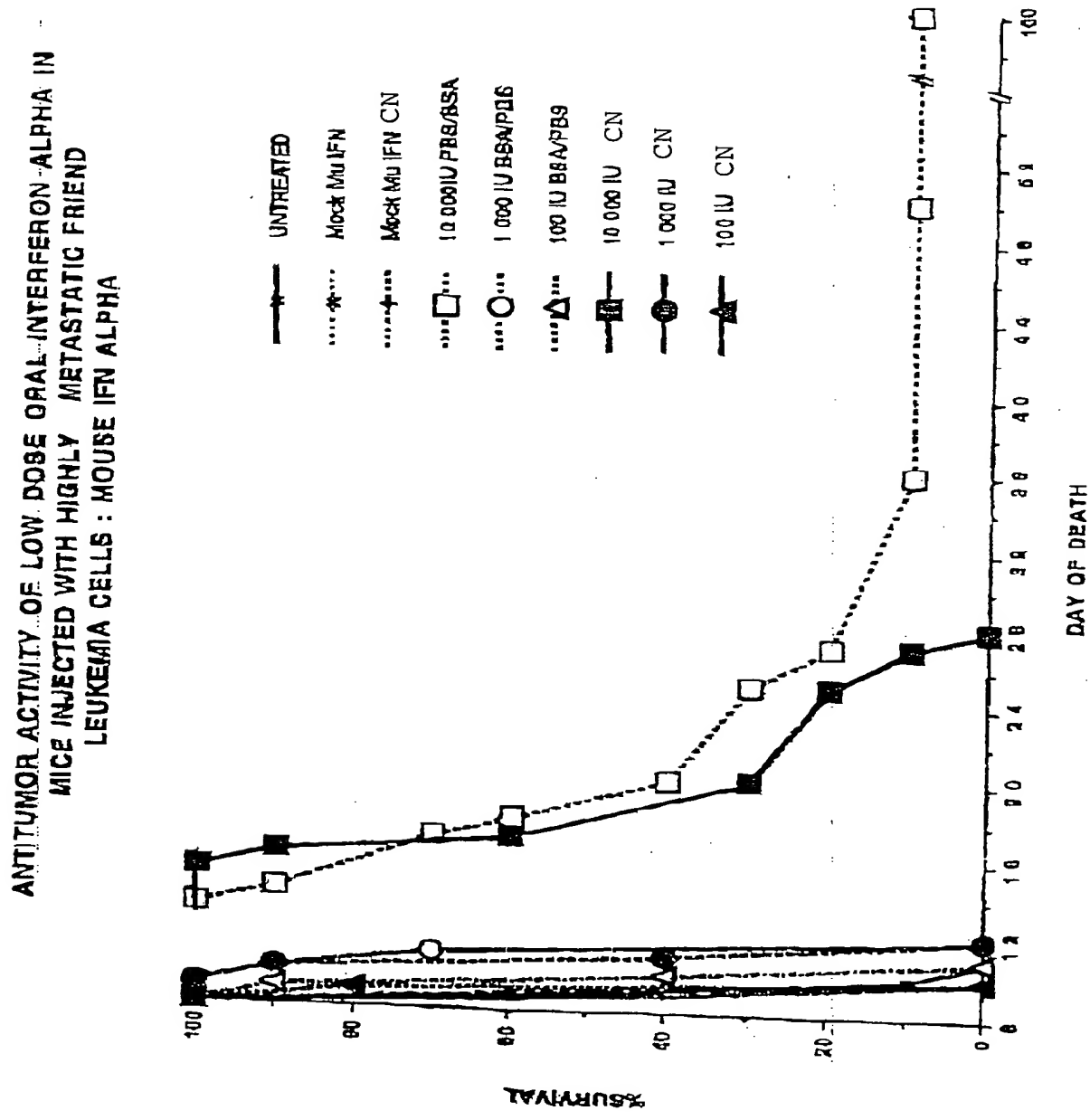
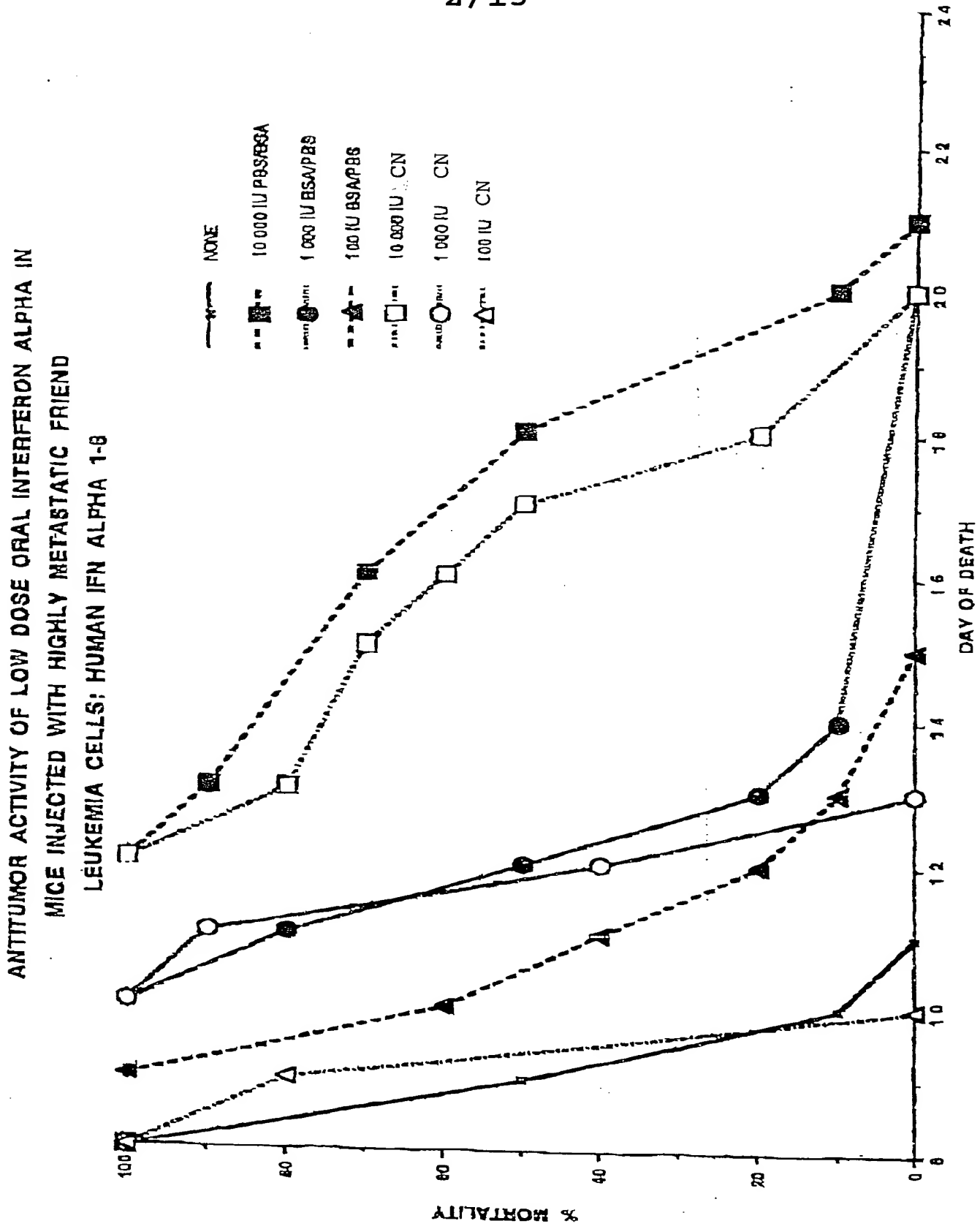

ANTITUMOR ACTIVITY OF LOW DOSE ORAL INTERFERON-ALPHA IN
MICE INJECTED WITH HIGHLY METASTATIC FRIEND
LEUKEMIA CELLS : MOUSE IFN ALPHA
UNTREATED
Mock Mu IFN
Mock Mu IFN CN
10 000 IU PBS/BSA
1 000 IU BSA/PBS
100 IU BSA/PBS
10 000 IU CN
1 000 IU CN
100 IU CN
%SURVIVAL
DAY OF DEATH

FIGURE 1A


ANTITUMOR ACTIVITY OF LOW DOSE ORAL INTERFERON ALPHA IN
MICE INJECTED WITH HIGHLY METASTATIC FRIEND
LEUKEMIA CELLS: HUMAN IFN ALPHA 1-8
NONE
10 000 IU PBS/BSA
1 000 IU BSA/PBS
100 IU BSA/PBS
10 000 IU CN
1 000 IU CN
100 IU CN
% MORTALITY
DAY OF DEATH
100
80
60
40
20
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6
8
10
12
14
16
18
20
22
24

FIGURE 1B

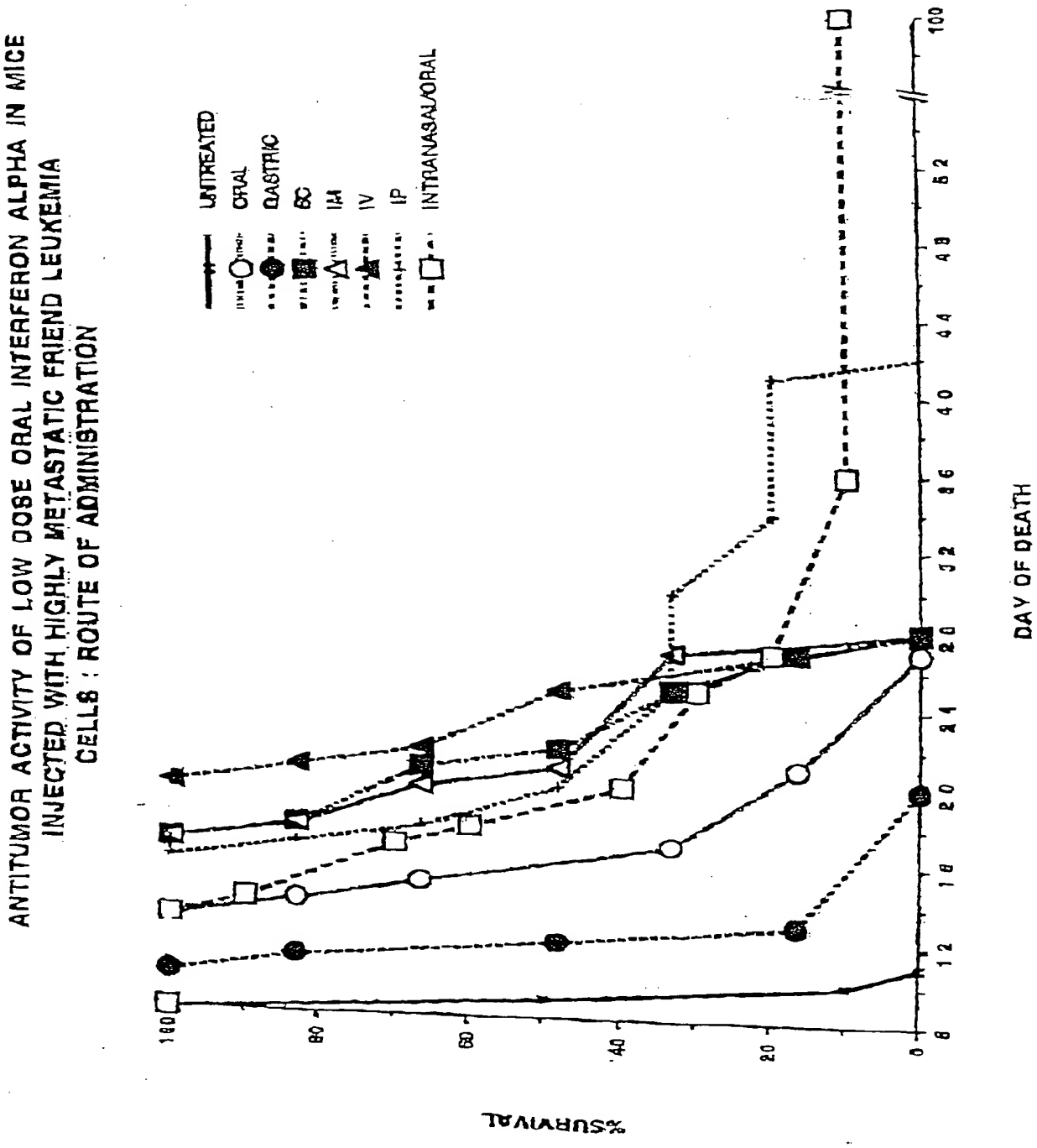
ANTITUMOR ACTIVITY OF LOW DOSE ORAL INTERFERON ALPHA IN MICE
INJECTED WITH HIGHLY METASTATIC FRIEND LEUKEMIA
CELLS : ROUTE OF ADMINISTRATION
UNTREATED
ORAL
GASTRIC
SC
IM
IV
IP
INTRANASAL/ORAL
%SURVIVAL
100
80
60
40
20
0
8
12
16
20
24
28
32
36
40
44
48
52
100
DAY OF DEATH

FIGURE 2

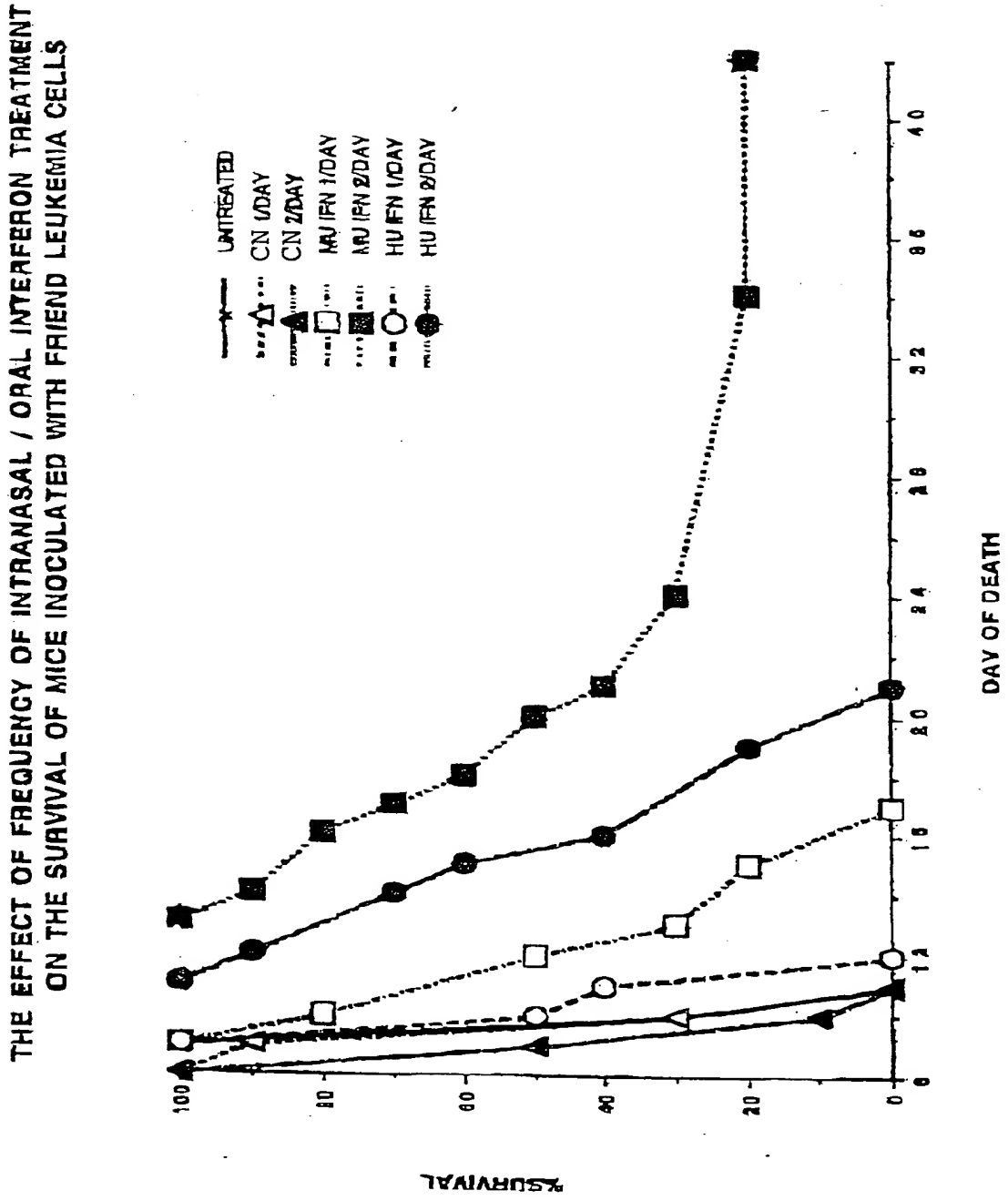
THE EFFECT OF FREQUENCY OF INTRANASAL / ORAL INTERFERON TREATMENT
ON THE SURVIVAL OF MICE INOCULATED WITH FRIEND LEUKEMIA CELLS
UNTREATED
CN 1/DAY
CN 2/DAY
MU IFN 1/DAY
MU IFN 2/DAY
HU IFN 1/DAY
HU IFN 2/DAY
%SURVIVAL
100
80
60
40
20
0
0
4
8
12
16
20
24
28
32
36
40
DAY OF DEATH

FIGURE 3

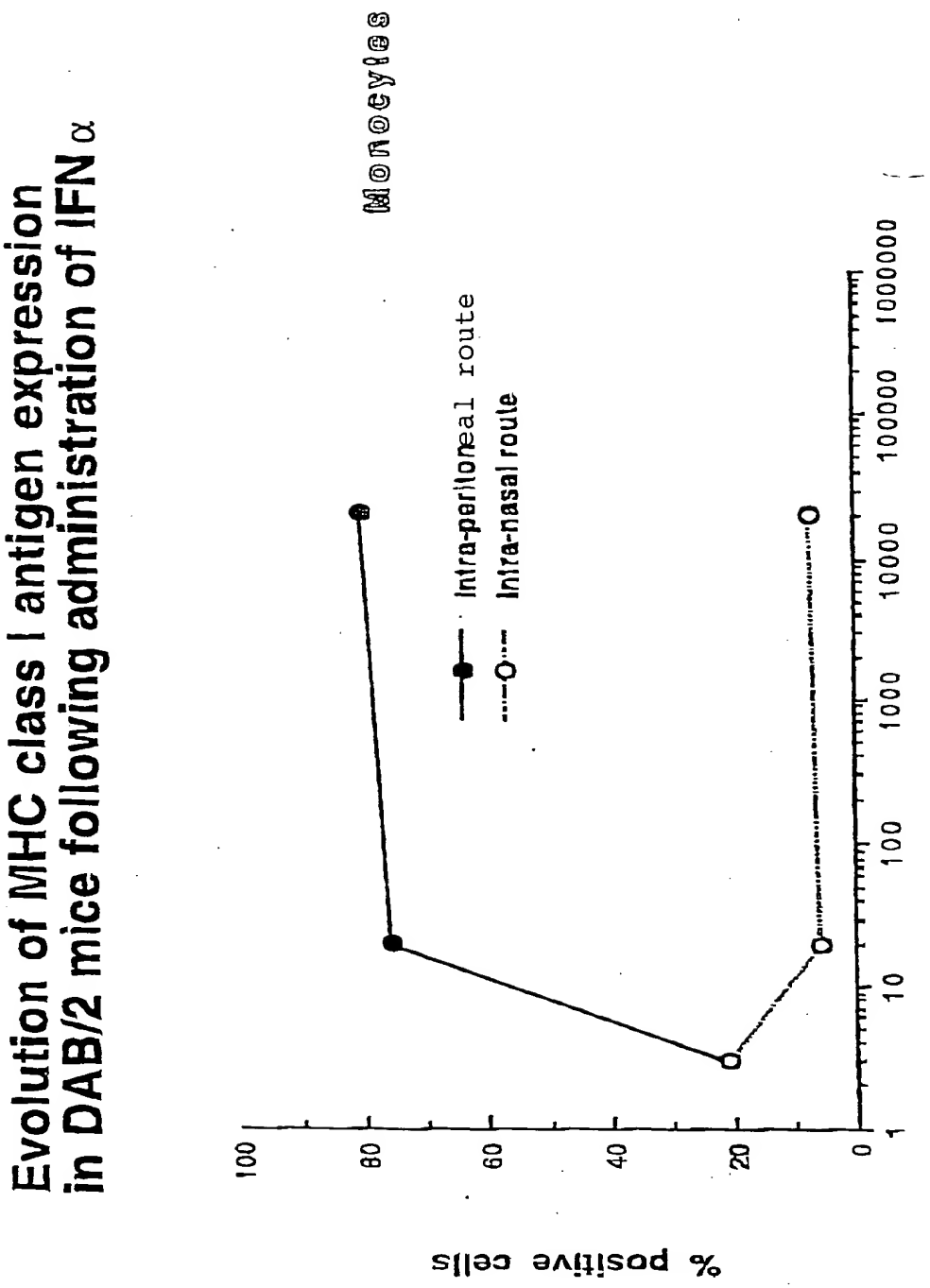
Evolution of MHC class I antigen expression
in DAB/2 mice following administration of IFN α
Monocytes
Intra-peritoneal route
Intra-nasal route
% positive cells
IFN α (IU/ml)
100
80
60
40
20
0
1
10
100
1000
10000
100000
1000000

FIGURE 4A

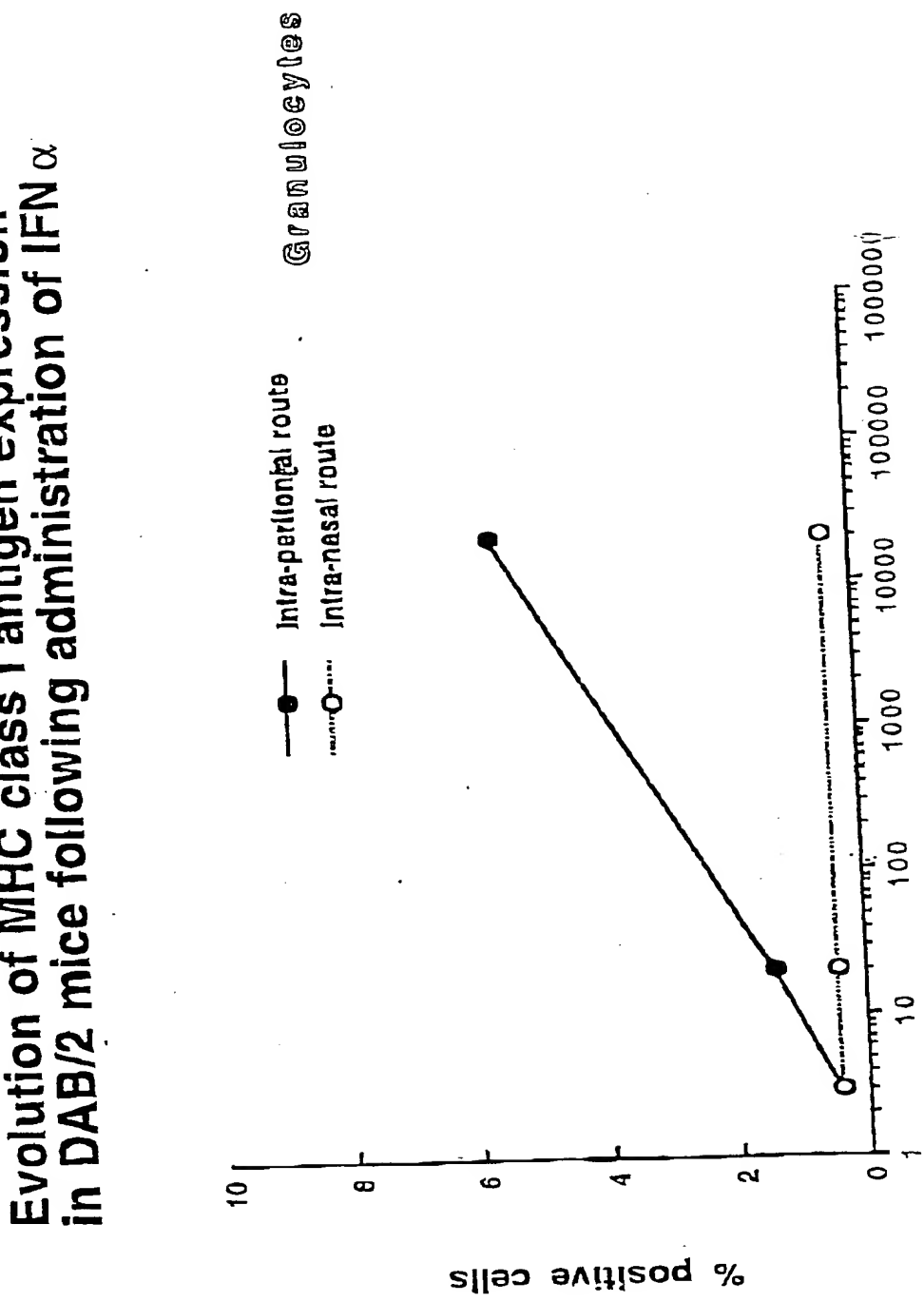
Evolution of MHC class I antigen expression
in DAB/2 mice following administration of IFN α
Granulocytes
Intra-peritonial route
Intra-nasal route
% positive cells
IFN α (IU/ml)
0
2
4
6
8
10
1
10
100
1000
10000
100000
1000000

FIGURE 4B

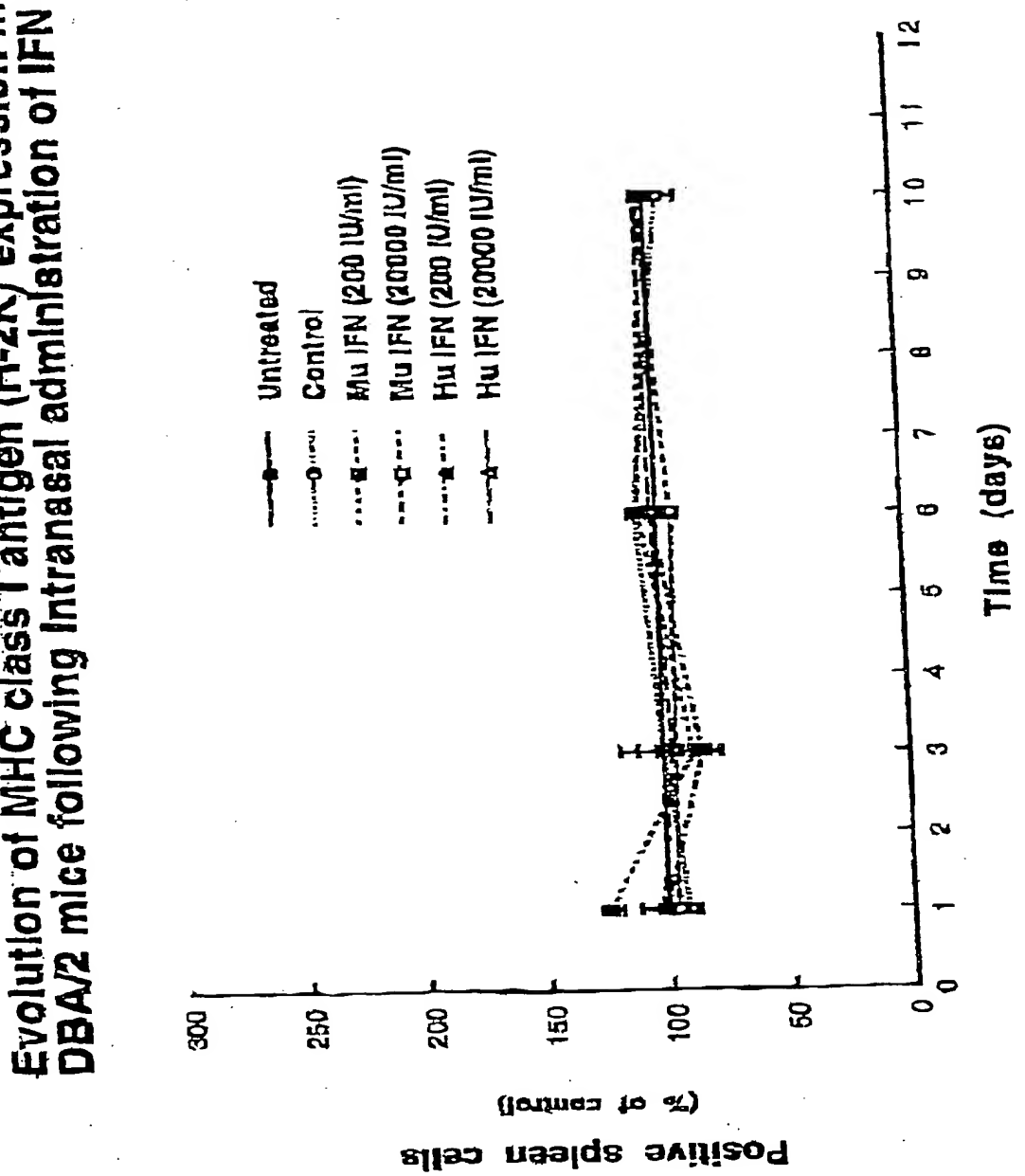
Evolution of MHC class I antigen (H-2K) expression in DBA/2 mice following Intranasal administration of IFN α
Untreated
Control
Mu IFN (200 IU/ml)
Mu IFN (20000 IU/ml)
Hu IFN (200 IU/ml)
Hu IFN (20000 IU/ml)
Positive spleen cells (% of control)
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (days)

FIGURE 5

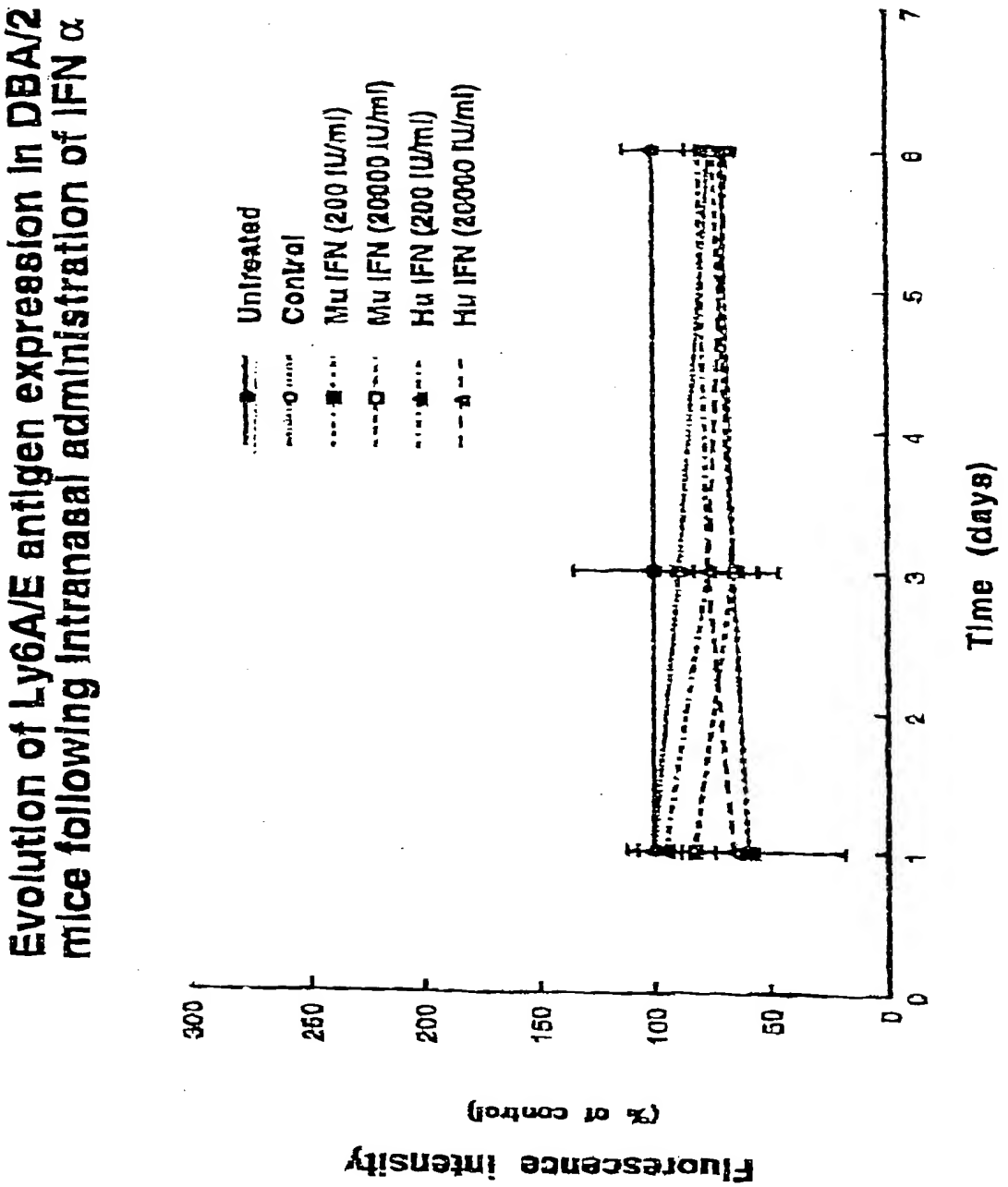
Evolution of Ly6A/E antigen expression in DBA/2 mice following intranasal administration of IFN α
Untreated
Control
Mu IFN (200 IU/ml)
Mu IFN (20000 IU/ml)
Hu IFN (200 IU/ml)
Hu IFN (20000 IU/ml)
Fluorescence intensity
(% of control)
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
Time (days)

FIGURE 6

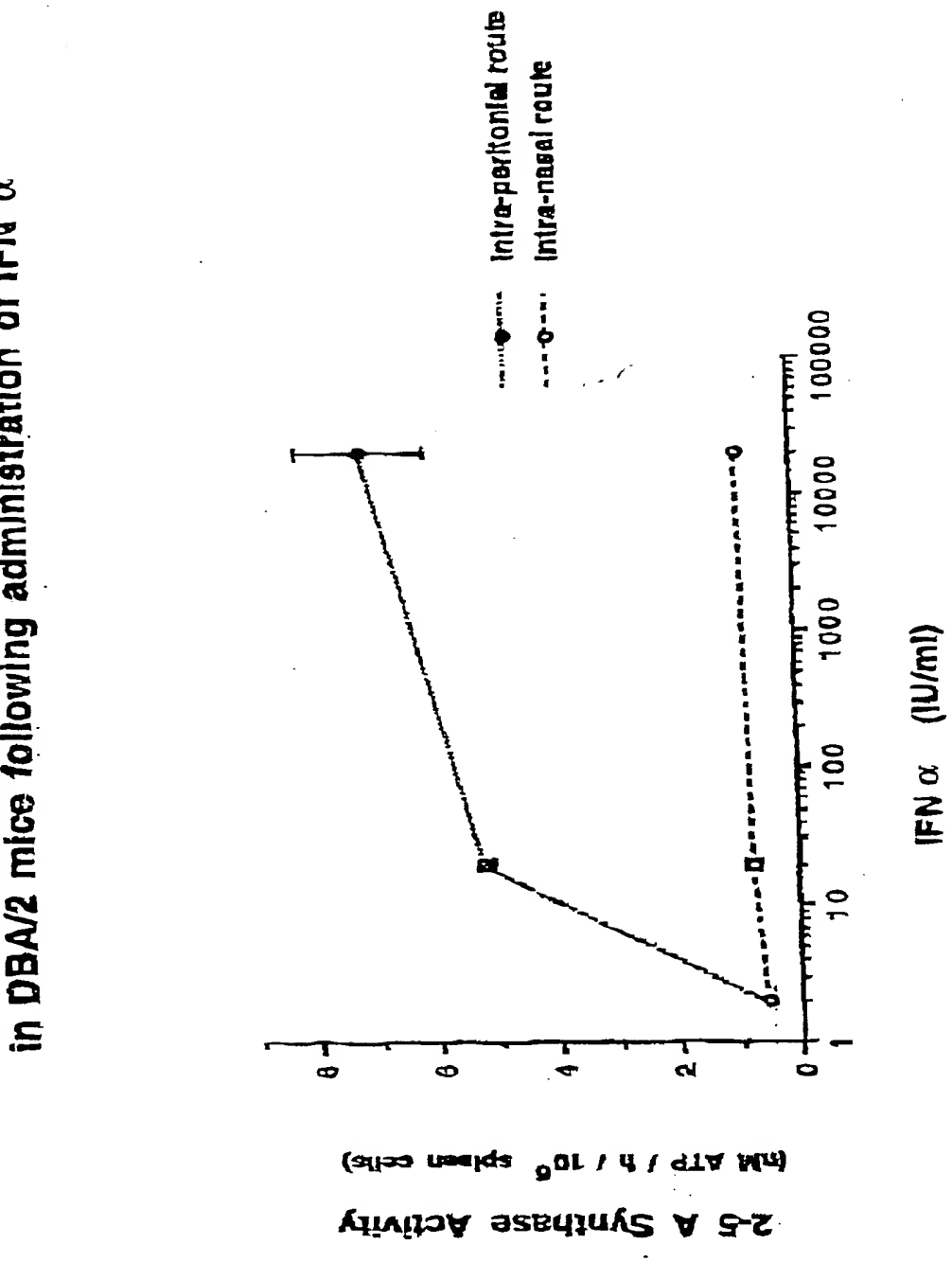
Induction of 2-5 A Synthase Activity
in DBA/2 mice following administration of IFN α
Intra-peritonial route
Intra-nasal route
2-5 A Synthase Activity
(fM ATP / h / 10⁶ spleen cells)
0
2
4
6
8
1
10
100
1000
10000
100000
IFN α (IU/ml)

FIGURE 7

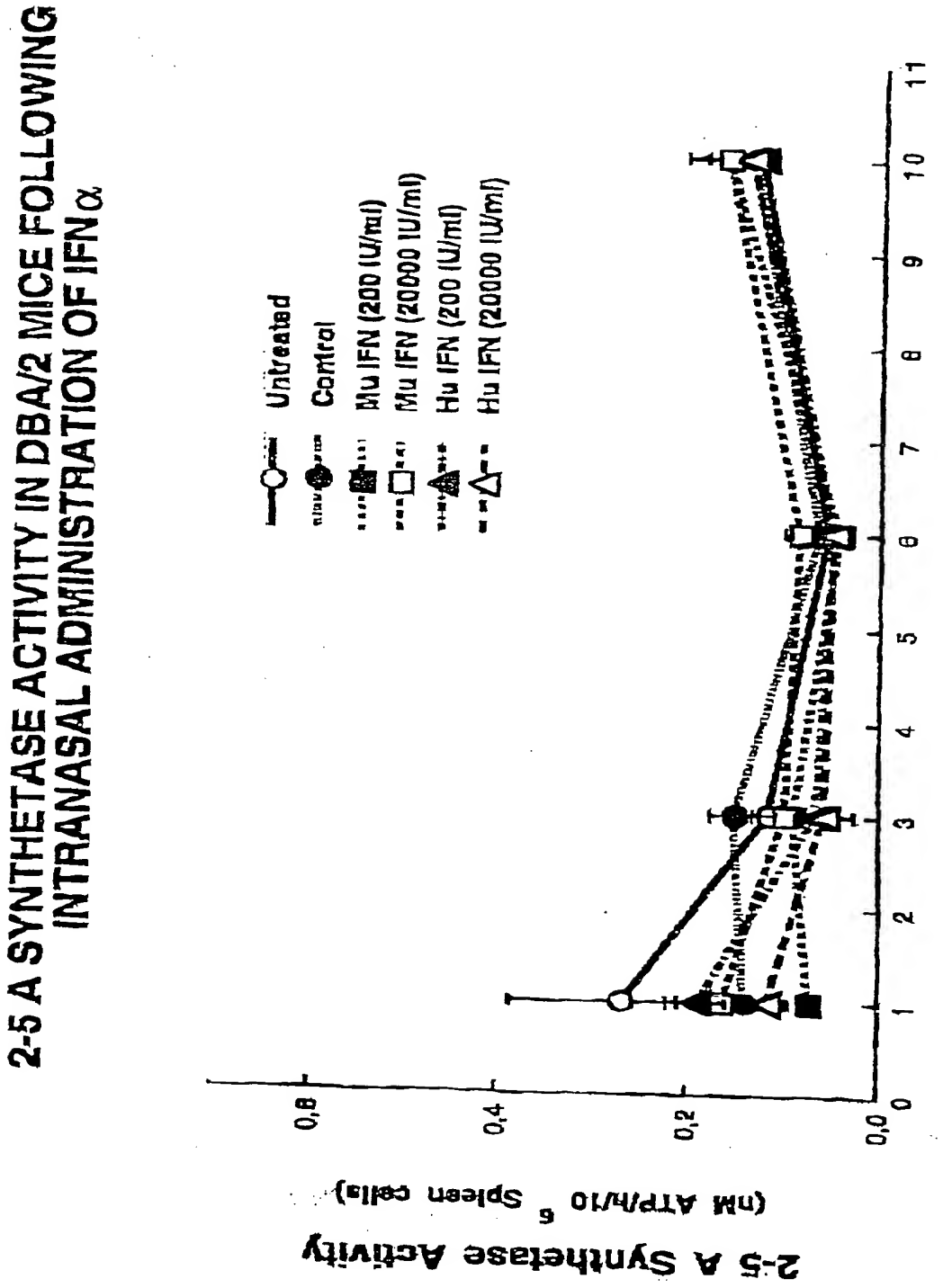
2-5 A SYNTHETASE ACTIVITY IN DBA/2 MICE FOLLOWING INTRANASAL ADMINISTRATION OF IFN α
Untreated
Control
Mu IFN (200 IU/ml)
Mu IFN (20000 IU/ml)
Hu IFN (200 IU/ml)
Hu IFN (20000 IU/ml)
2-5 A Synthetase Activity
(nM ATP/h/10 6 Spleen cells)
0,0
0,2
0,4
0,8
0
1
2
3
4
5
6
7
8
9
10
11
Time (days)

FIGURE 8

IFN TITERS IN THE SERUM OF SWISS MICE TREATED WITH I-125 IFN

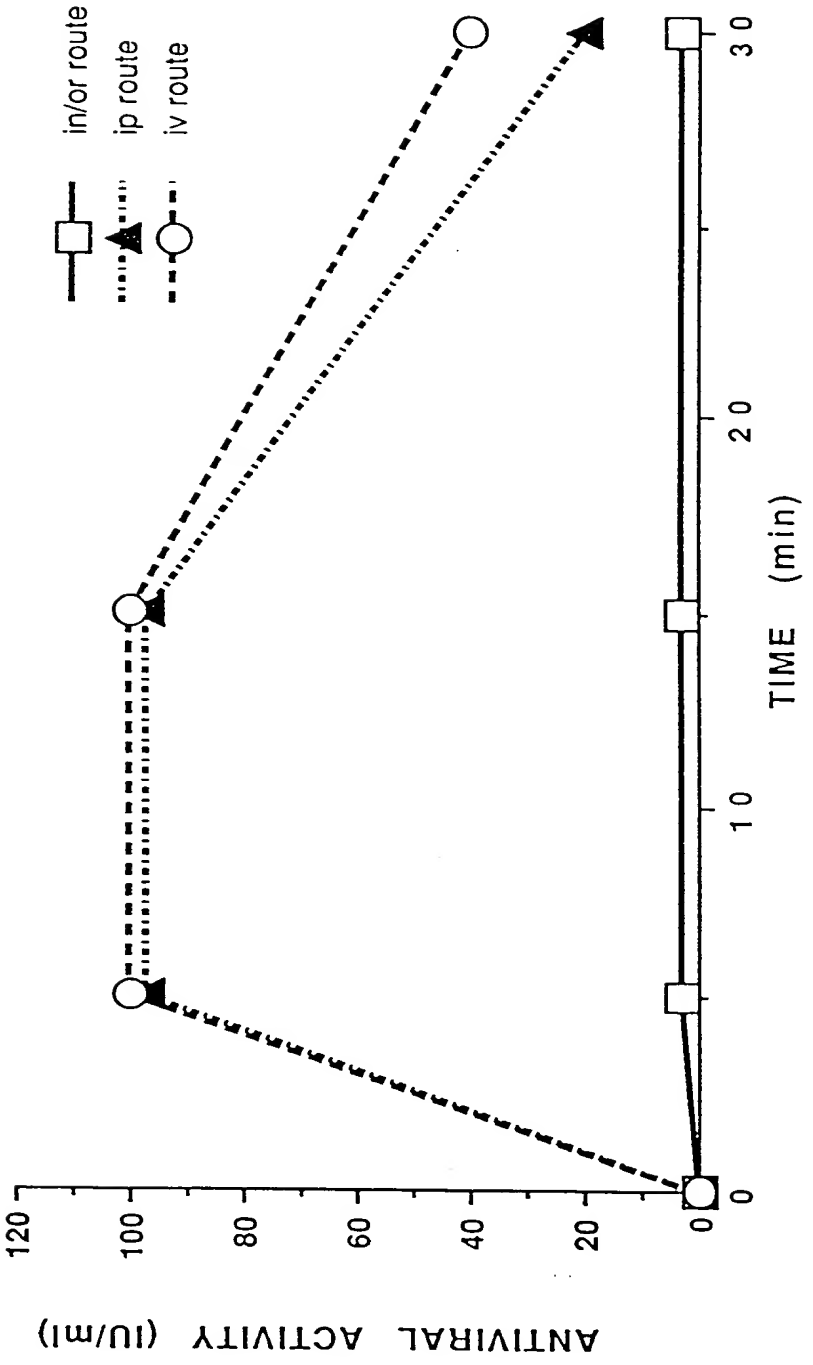

FIGURE 9

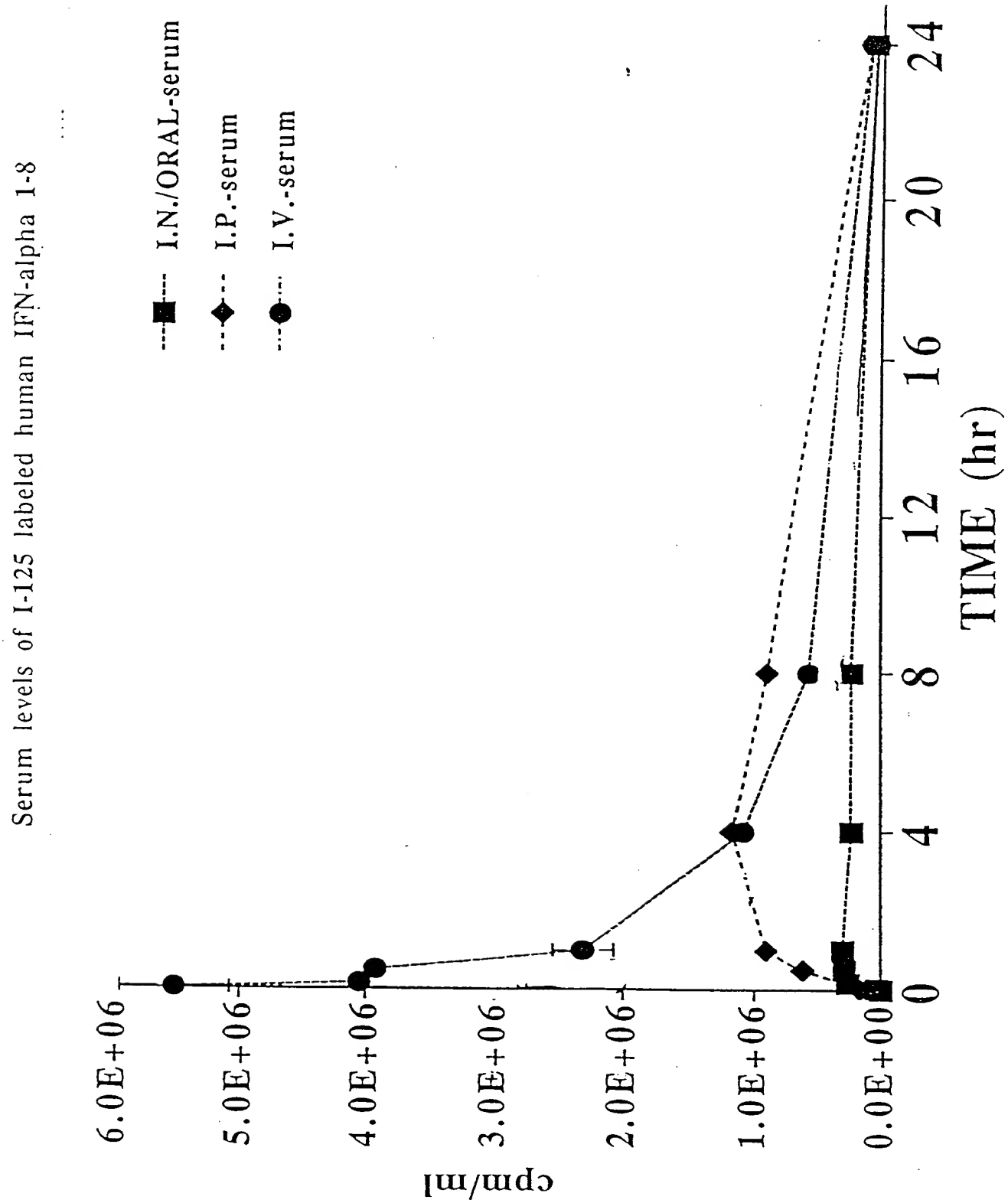
Serum levels of I-125 labeled human IFN-alpha 1-8
I.N./ORAL-serum
I.P.-serum
I.V.-serum
cpm/ml
6.0E+06
5.0E+06
4.0E+06
3.0E+06
2.0E+06
1.0E+06
0.0E+00
0
4
8
12
16
20
24
TIME (hr)

FIGURE 10

Whole blood and serum levels of intranasally administered I-125 labeled human IFN-alpha 1-8

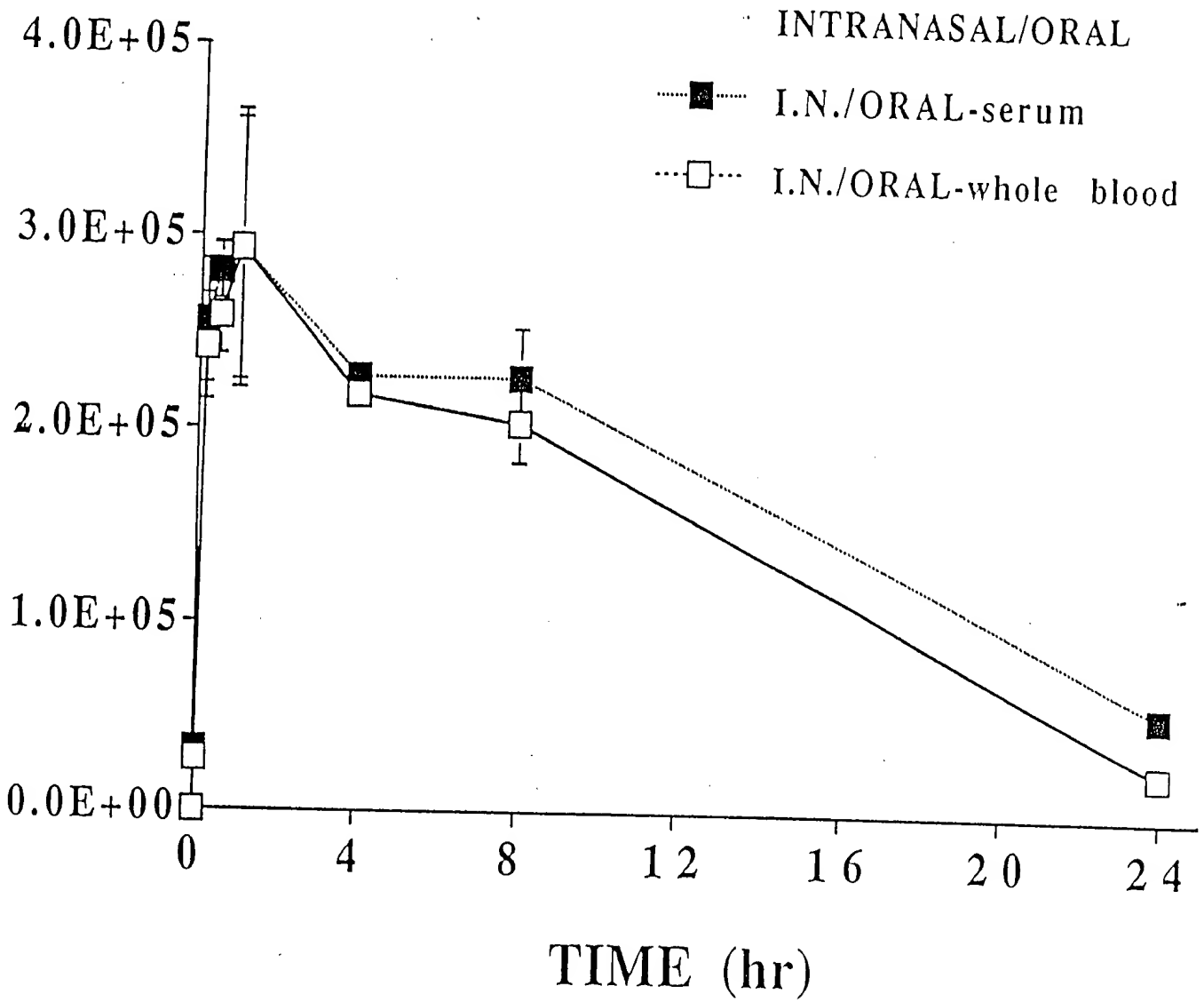

FIGURE 11